FACTORS AFFECTING PREIMPLANTATION EMBRYONIC MORTALITY IN DAIRY CATTLE EXPOSED TO ELEVATED ENVIRONMENTAL TEMPERATURES

By

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Early embryonic mortality may be the major factor responsible for summer-time depression of pregnancy rate in cattle. Holstein heifers exposed to acute heat stress prior to ovulation or to daily intermittent heat stress during the first 7 days of pregnancy had a higher incidence of abnormal and retarded embryos. The preovulatory oocyte and early cleavage stage embryo are extremely sensitive to maternal hyperthermia, possibly due to direct effects of high temperature on biochemical processes within the embryo. Heat stress in vitro reduced incorporation of [^{35}S]methionine into proteins by Day 7 embryos and induced synthesis of a 70 kDa heat shock protein (hsp). Synthesis of hsp by embryos suggests that, by Day 7 of pregnancy, embryos have acquired some degree of thermotolerance and are able to survive acute increases in uterine temperature.

Embryo transfer at Day 7 post estrus may bypass the period of embryonic sensitivity to maternal hyperthermia. Pregnancy rates of summer heat-stressed recipient dairy cows were higher compared to those of artificially inseminated (AI) cows. Embryo transfer may provide an alternative to AI to partially circumvent heat-induced infertility in cattle.

Embryos continue to be sensitive to heat beyond Day 7 of development. In vitro heat stress of Day 17 conceptuses reduced incorporation of [^{3}H]leucine into proteins and induced synthesis of hsps. Secretion of PGF by endometrial explants of pregnant cows increased in response to heat. This was due to partial inactivation of an endometrial prostaglandin synthesis inhibitor (EPSI). In vivo, summer heat stress increased oxytocin-induced concentrations of PGFM in non-pregnant dairy cows. Decreased responsiveness of the pregnant uterus to an induced secretion of PGF suggests that presence of a conceptus attenuates uterine responsiveness to heat stress.

Exposure of pregnant cows to high temperature retards embryonic development and may disrupt the balance between conceptus and endometrial factors required for maintenance of the corpus luteum during early pregnancy.

CHAPTER 1
REVIEW OF LITERATURE

Introduction

Environmental heat stress often results in seasonal periods of infertility in dairy cattle. In tropical and subtropical areas of the world, pregnancy rates following natural or artificial insemination range as low as 10 to 15 % during stressful months of the year. Although various reproductive processes may be affected adversely by an unfavorable thermal environment, early embryonic mortality may be the major factor causing summer-time depression of pregnancy rate in cattle.

Successful preimplantation embryonic development and maintenance of pregnancy represents the culminated results of numerous and complex endocrinological, morphological, physiological, biochemical and immunological interactions between the conceptus and mother. An understanding of the physiological processes involved in early embryonic development and pregnancy recognition will provide the reader with a basis for understanding the various biological processes, adversely affected by environmental stress, which compromise the well being of the embryo in its oviductal or uterine environment.

Anatomy and Physiology of Preimplantation Bovine Embryos

Oocyte Maturation and Early Embryo Development

Maturation of the oocyte within its preovulatory large antral follicle involves a complex series of events that renders the oocyte capable of successful fertilization and preimplantation embryonic development. Oocyte maturation is commonly considered to be the developmental progression of nuclear and cytoplasmic maturation from the appearance of the germinal vesicle of the arrested dictyate stage, through meiosis I to metaphase II and extrusion of the first polar body (Hyttel et al., 1986; Moor and Gandolfi, 1987). Additionally, oocyte maturation is accompanied by quantitative and qualitative alterations in the synthesis of RNA (Rodman and Bachvarova, 1976) and proteins (Wassarman et al., 1981). Proteins within the cytoplasm of the activated oocyte regulate events such as meiotic maturation, formation of the male pronuclear envelope, initiation and completion of cleavage-stage embryo development (First and Haseltine, 1987; Moor and Gandolfi, 1987). For example, immature pig oocytes cultured in a medium supplemented with cycloheximide, to block protein synthesis, were unable to undergo activation due to a complete block of germinal vesicle breakdown (Fulka et al., 1986). Additionally common defects resulting from fertilization of immature follicular oocytes, which have not resumed active protein synthesis, include failure to progress to organized blastocyst stage embryos (Staigmiller and Moor, 1984; Leibfried-Rutledge et al., 1987). Since the transcription of embryonic products required for successful compaction and blastocoele formation (Kidder and McLachlin, 1985) occurs well in advance of these events, i.e., during oocyte maturation, failure of the embryo to pass through these particular stages in vivo, may be due to adverse environmental conditions experienced earlier in oocytic or embryonic development.

In the bovine, the events associated with nuclear maturation have been chronicled. Upon release from antral follicles (>1.8 mm) at the time ovulation, cumulus-oocyte complexes undergo spontaneous nuclear maturation (Edwards, 1965; Motlik and Fulka, 1976) within the oviduct, requiring approximately 24 h to complete the first meiotic division (Edwards, 1965; Leibfried and First, 1979). Defining the day of estrus as Day 0, fertilization of the oocyte is completed on Day 1, in the area of the ampullary-isthmic junction of the oviduct. Surrounding the one-cell fertilized ova is a thick mucopolysacharide layer called the zona pellucida. Transmission electron microscopy of the cleaving bovine embryo (Flechon and Gwatkin, 1980), shows the zona pellucida to be a permeable, acelular structure, more spongy in both its inner and outer surfaces than in its center. It is traversed by channels left by the processes of the corona radiata cells which previously made contact with the oocyte. The zona pellucida is essential to the normal development of bovine embryos up to the fully compacted morula stage. The zona pellucida may play a role in conserving the microenvironment of the perivitelline space surrounding the embryo.

The first cleavage of the zygote into two blastomeres occurs rapidly, usually within 24 to 28 h post ovulation (Thibault, 1966). Embryos are described according to cell number up to the 16- to 32-cell stage, at approximately 4 to 5 days of development, at which point they are referred to as morulae. At this time most have passed from the oviduct into the uterus. Although individual blastomeres of the early morula remain readily distinguishable, they begin to coalesce and lose their sharp outlines by about the 32-cell stage.

During early cleavage stages of development, embryos undergo a multitude of distinct morphological and physiological changes. Individual blastomeres within the bovine embryo have rounded contours except where they touch one another. Their plasma membranes are covered with microvilli even in regions of blastomere contact (Brackett et al., 1980, see Betteridge and Flechon, 1988). At the 8- to 16-cell stage of development, blastomeric plasma membranes form regionalized tight junctions between adjacent blastomeres (Betteridge and Flechon, 1988). This calcium-dependent (Hyafil et al., 1981) adhesion process between blastomeres establishes a permeability seal on the outside of the embryo and is generally regarded as a prerequisite for the formation of a fluid filled blastocoele mainly later in development.

Transmission electron microscopy of the cleaving bovine embryo (Flechon and Gwatkin, 1980) show the cytoplasm within individual blastomeres to be characterized by the presence of numerous large vesicles, very large lipid inclusions, primitive mitochondria containing few cristae, and organelle-free zones in cortical areas of the cytoplasm (Betteridge and Flechon, 1988). Nucleoli within the nuclei are dense in the early cleavage stages (Crozet, 1984; King et al., 1987), but by the 8- to 16-cell stage, nucleoli become vacuolated and lose their compact appearance (King et al., 1987; Camous et al., 1986) coincident with activation of the embryonic genome.

Early cleavage spans a unique period of embryonic development when control of development passes from the maternal to the embryonic genome (King et al., 1985). In cattle, activation of the embryonic genome and transition from a maternally-directed to embryo-directed phase in protein synthesis occurs at approximately the 8- to 16-cell stage of embryo development. Evidence confirming the timing of this event comes from studies of (1) the nucleolus organizer regions of bovine chromosomes; (2) the cytological and ultrastructural analysis of nucleoli; and (3) the resumption of mRNA and rRNA synthesis in 8-cell embryos (King et al., 1987; Camous et al., 1986; Pivko et al., 1986;

The phase of embryonic development associated with activation of the embryonic genome is particularly vulnerable to adverse environmental conditions. This is reflected by an 8- to 16-cell block in embryo development in vitro and even a delay in the rhythm of cleavage in vivo (Newcomb, 1982; Eyestone and First, 1986). It has been suggested that, as yet undefined factors present in vivo, such as provided by various oviductal-embryo co-culture systems, are necessary to overcome this in vitro developmental block (Camous et al., 1984; Gandolfi and Moor, 1987). A better understanding of these factors should eventually permit better duplication of the oviductal milieu in culture systems.

The transition from compact morula to blastocyst occurs between Days 6 and 8 of gestation, at which time the early blastocyst, about 160 um in diameter (Linares and King, 1980), still remains enclosed in a zona pellucida. At the early blastocyst stage, the embryo contains about 100 cells (Mannaerts, 1986) comprising the inner cell mass (ICM) and the trophectoderm. The fully expanded blastocyst, with a very obvious blastocoele and thinned zona pellucida, contains about 160 cells (Mannaerts, 1986).

Development of Preimplantation Stage Embryos

Approximately 9 days after estrus the bovine embryo undergoes the hatching process and emerges from the zona pellucida. The mechanism by which the blastocyst effects a partial lysis of the zona pellucida and facilitates its own hatching may involve activation of intracellular plasminogen to the enzyme plasmin (Menino and Williams, 1987).

Soon after hatching, the blastocyst begins to rapidly divide and increases in cell number are highly correlated with the diameter of the spherical blastocyst (Renard et al., 1978). In the largest spherical blastocysts, usually about 375 um in diameter, found on Days 11 to 12 post estrus, the ICM accounts for less than 25 % of the approximately 1000 cells (Renard et al., 1978). After Day 12 the shape of the blastocyst changes from spherical to ovoid, marking a transitionary phase which precedes elongation that usually begins between Days 12 and 14 (Betteridge et al., 1980).

Ultrastructural studies (Massip et al., 1981; Linares and Ploen, 1981) have shown that endodermal cells spread out from beneath the ICM at about Day 8, and by Day 10 completely line the trophectodermal cells that surround the blastocoele, thereby forming the trophoblast. Mesodermal cells, which also differentiate from the ICM, begin to migrate out between the trophectoderm and endoderm at about Days 14 to 16 (Greenstein and Foley, 1958a,b), and, as they do so, separate into two mesodermal layers. The outer layer of mesoderm lines the trophectoderm to form the chorion while the inner layer covers the endoderm to constitute the wall of the yolk sac.

The period of bovine conceptus development between Day 16 and attachment to the maternal uterine caruncles (Days 20 to 22) is characterized by rapid growth and development of both the ICM (the embryo proper) and the trophoblast (extraembryonic membranes). Between Days 16 and 17, the trophoblast takes on a filamentous ribbon-like appearance (Chang, 1952). Lengths of the trophoblastic extraembryonic membranes are tremendously variable among conceptuses during this time period, with reports from 2 to over 500 mm (Hawk et al., 1955; Betteridge et al., 1980) The conceptus grows rapidly and occupies nearly two-thirds of the gravid uterine horn by Day 18 of pregnancy and between Days 19 and 20, conceptus membranes extend throughout the gravid and into the contralateral non-gravid uterine horn. Rapid expansion of extraembryonic membranes continues such that, by days 24 to 25 of gestation, membranes extend to the tip of the non-gravid uterine horn

Bovine trophoblast elongation is essential (1) to insure increased surface area for nutrient uptake during transition from yolk sac to chorioallantoic nutrition; and (2) to increase uterine area affected by conceptus products. Substances produced by the conceptus in utero may act in a paracrine fashion to exert effects locally on endometrial or other uterine tissues and/or in and endocrine fashion where conceptus products enter the maternal circulation to affect target tissues (Krieger, 1982).

Histologically and cytologically the trophoblast surface at Day 18 consists primarily of a single layer of roughly cuboidal trophoblastic cells with an inner lining of flattened endodermal cells (Greenstein et al., 1958). Well-developed spot desmosomes link the adjacent cell borders in both the trophoblast and endodermal layers (Carnegie et al., 1985). The trophoblast cells contain acid phosphatase-positive, lysosome-like organelles, the mean diameter of which increases greatly prior to conceptus attachment. By Day 17 of gestation, trophoblast cells accumulate vast amounts of lipid, and periodic acid-Schiff-positive binucleate cells appear within the trophoblast layer (Greenstein et al., 1958).

Trophoblastic apical microvilli (Guillomot and Guay, 1982) cover the trophoblastic cell surface and may be involved in the absorption of extracellular products by the conceptus prior to attachment to the uterus as suggested by the presence of vesicles attached to the apical microvilli, endocytotic invaginations of the plasma membrane, and dense bodies within the cytoplasm (Guillomot and Guay, 1982). Additionally, ultrastructural appearance of Day 17 endometrial epithelium was indistinguishable in pregnant versus cyclic cattle (King et al., 1981), suggesting that no physical interaction between conceptus and endometrium had occurred at this stage of pregnancy.

The bovine conceptus begins to attach to the uterine epithelium by Days 20 to 22 of gestation (King et al., 1980, 1981) and this event is associated wth trophoblastic cell proliferation on portions of the conceptus facing uterine glands (Guillomot and Guay, 1982). Implantation of the conceptus in the uterus is non-invasive in that the trophoblast does not erode the uterine epithelium, but attaches by interdigitation of apical microvilli on opposing surfaces of trophectodermal cells and underlying epithelia of the endometrium. It is only after Day 20 of gestation that placentome formation begins in the cow. For this reason, the conceptus is believed to rely upon endometrial glandular secretions (uterine histotrophe) for nutritional maintenance for a considerable portion of early pregnancy (Bazer et al., 1981a)

Metabolic Activity of Preimplantation Embryos

During preimplantation embryonic development there, is a gradual change in energy metabolism and substrate requirements (see Brinster, 1973). Initially, the oocyte utilizes primarily pyruvate as an energy source (Brinster, 1967b) and glucose oxidation is very low (Boone et al., 1978). However, as development progresses to the morula/blastocyst stage and the embryonic genome is activated, energy source requirements and energy metabolism change and are apparently similar to those of most adult cells. Thus glucose, is metabolized by blastocysts which also produce lactate (Renard et al., 1980). Using in vitro culture systems capable of measuring the metabolism of individual embryos, studies of energy requirements have been considerably extended (Rieger, 1984). These studies, with bovine embryos, have demonstrated that at Day 7 the Krebs cycle is active but the Embden-Meyerhof pathway is blocked and glucose is metabolized via the pentose phosphate shunt.

Metabolic activity of the bovine embryo is essential to provide energy required for cell division and to synthesize products that are secreted into the uterine lumen that affect the maternal system in ways that are just beginning to be understood. The range of biochemical products produced by cleavage stage embryos that have been investigated is limited. Embryos produce a phosphoglyceride platelet activating factor (PAF) which is evidently essential for the establishment of pregnancy in mice, and induces the expression of an immunosuppressive early pregnancy factor (EPF) (O'Neill, 1987; Hanahan, 1986; Orozco et al., 1986). Recent indications that bovine embryos produce EPF (Shaw and Morton, 1980; Nancarrow et al., 1981; O'Neill, 1987) and a lipid-like luteotrophic substance that may be analagous to PAF (Hickey and Hansel, 1987) has spurred a great deal of interest in developing assays to detect these products as a potential route to early pregnancy diagnosis (Shaw and Morton, 1980; Wesser and Rader, 1987). Continued research involving PAF and EPF will assuredly further our understanding of embryonic-induced events associated with successful embryonic development and maternal recognition of pregnancy in cattle.

Protein Synthesis

Active protein synthesis in all cell types of the elongated blastocyst can be inferred from the large number of intracellular ribosomes and the presence of rough endoplasmic reticulum. The periattachment-stage bovine conceptus produces a complex array of proteins and polypeptides in vitro (Bartol et al., 1985). These proteins are primarily low molecular weight (20 to 26 kDa) acidic (pI 5.6 to 6.5) proteins. This family of polypeptides consists of prominent but transient products, produced by conceptuses from Day 16 to Day 24 post-estrus. The temporal pattern of appearance and the transient nature of production of such polypeptides suggests that such products may serve important functional roles during the period associated with establishment of pregnancy. An indepth discussion of the role of conceptus secretory proteins in maternal recognition of pregnancy will be presented in a subsequent section of this chapter.

The number and/or amount of polypeptides produced in vitro and released into culture medium by bovine conceptuses increases after Day 16 of gestation. The time between Days 16 and 19 of gestation constitutes a dynamic period of rapid elongation of the trophoblast, establishment of a functional but temporary yolk sac and vitelline circulation, initial expansion of the allantois, and appearance of large binucleate cells on the external trophoblast surface (Chang, 1952; Greenstein and Foley, 1958a; Greenstein et al., 1958;

King et al., 1980; Wooding and Wathes, 1980). Hence, in vivo, an array of tissues and cell types contributes to production of proteins by conceptuses during this period.

In addition to proteins already known to be important for the establishment of pregnancy, such as bovine trophoblast protein-1 (Helmer et al., 1987), products that may be of use for early diagnosis of pregnancy, such as 'protein B' (Reimers et al., 1985; Sasser et al., 1986; Sasser and Ruder, 1987), or which have known functions in other species, such as alpha-fetoprotein, are of particular interest. Alpha-fetoprotein, which has immunosuppressive properties, is synthesized as early as Day 14 by the bovine blastocyst and can be detected in allantoic fluid by Day 16 post-estrus (Janzen et al.,1982). This protein appears to be sequestered in significant amounts within the uterine lumen between Days 14 and 46 of pregnancy, where it may possibly exert an immunoprotective effect on the developing conceptus. Additionally, alpha-fetoprotein may regulate availability of uterine luminal steroids. Rat alpha-fetoprotein was reported to bind both testosterone and estradiol with equal affinity ($K_d = 1 \times 10^{-10}$M, Clark et al., 1977).

Bovine placental lactogen (bPL) can be detected by radioreceptor assay in homogenates of bovine conceptuses from Days 17, 24 and 25 of gestation (Flint et al., 1979). It was suggested that synthesis of bPL by Day 17 conceptuses was related to the appearance of trophoblast binucleate cells, which differentiate in bovine trophectoderm by Day 16 (Flint et al., 1979; Greenstein et al., 1958). Placental lactogen may have important functions within the conceptus. Carnegie et al. (1982) observed that ovine PL first appeared after Day 12 post estrus, coincident with blastocyst transformation from a spherical to filamentous form, suggesting that oPL might be related to events associated with blastocyst elongation and water movement across extra-embryonic membranes. Indeed, human PL elicited changes in both short-circuit current and potential differences of porcine chorioallantoic membranes in vitro, which were consistent with prevention of water movement from fetal to maternal components (Bazer et al., 1981b).

Prostaglandin Synthesis

Prostaglandins are produced and metabolized by the conceptus. Bovine blastocysts recovered at 16 and 19 days of pregnancy synthesized considerable amounts of prostaglandins in vitro (Lewis et al., 1982) with both PGF and PGE_2 production increasing with day of gestation. Similarly, Shemesh et al. (1979) reported age dependent increases in measurable amounts of immunoreactive PGF and PGE_2 in conceptus tissues collected on Days 13, 15 and 17 post estrus. Lewis et al. (1982), examining the ability of bovine conceptuses from Days 16 and 19 to metabolize arachidonic acid (AA), reported incorporation of substantial amounts of radiolabeled AA into homogenized blastocyst tissues.

To some degree, conceptus prostaglandins appear to be sequestered and/or compartmentalized within the uterine lumen. For example, PGF and PGE_2 contents of uterine flushings on Days 16 and 19 post-estrus from pregnant cattle were greater than those of cyclic or non-pregnant cattle (Bartol et al., 1981a). The physiological function of conceptus produced prostaglandins in the growth and development of bovine blastocysts has been speculated. Increases in blood flow to the pregnant uterine horn at Day 16 (Ford et al., 1979) may be due to conceptus production of vasodilatory agents such as PGE_2. A selective increase in uterine blood flow may increase histotropic nutrients for the developing conceptus.

Steroid Synthesis

Steroid hormones are synthesized and metabolized by the bovine conceptus and represent another molecular class of conceptus signals that may be involved in the process of pregnancy recognition. Bovine conceptuses recovered on Days 13, 15 and 16 of pregnancy produce progesterone (P4), some testosterone (T) and limited quantities of estradiol-17 (E2-17) during incubation (Shemesh et al., 1979). Eley et al. (1983) also found conversion of radiolabeled androstenedione to estrone and E2-17 by Day 15 to 17 bovine blastocysts. Furthermore, there is extensive metabolism by the Day 13 to 27 bovine conceptus of radiolabeled testosterone (Chenault, 1980) and P4 (Eley et al., 1983) to

5β-reduced metabolites such as pregnanes, androstanes and hydroxylated products

The 5β-reduced metabolites evolving from the 5β-reductase and hydroxy steriod dehydrogenase enzyme systems of the conceptus may be involved in stimulating erthropoietic activity (Gorshein and Gardner, 1970) and hemoglobin synthesis (Necheles and Rai, 1969) in tissues forming blood cells. This could be important for the developing embryo, which begins to form a circulatory system on Day 18 of gestation. Furthermore, certain 5β-reduced pregnanes suppress myometrial uterine contractility in vitro (Kubli-Garfias, 1979) and perhaps, in utero, these steroid metabolites alter membrane function and stabilize target cells within either the endometrium or myometrium.

Oviductal-Uterine Environment of Preimplantation Embryos

Pregnancy depends upon a series of appropriate coordinated interactions between the developing conceptus and mother. The maternal reproductive system, must provide an appropriate fluid medium for oocyte fertilization and early cleavage stages of embryo development within the oviduct. The uterine lumen constitutes the environment of the bovine embryo prior to implantation and placentation, implying that the uterine environment and, in particular, the milieu of micro- and macro-molecular components of uterine secretions are vitally concerned with continued conceptus develop and survival beyond the blastocyst stage. A strict requirement for synchrony between donor and recipients in bovine embryo transfer (Sreenan, 1978; Seidel, 1981), to maintain high rates of successful pregnancy, emphasizes the importance of the uterine environment.

Oviductal Microenvironment

The oviduct provides a unique environment that is beneficial or even essential for sustaining embryonic development up to approximately the 8- to 16-cell stage before embryos are transported into the uterus. Embryos removed from the oviduct, or produced as a result of in vitro fertilization tend to exhibit delayed and/or arrested development when maintained in culture medium alone (Eyestone and First, 1986). However, if fertilized 1-cell embryos are transferred to the oviduct in vivo, embryonic development progresses at a normal rate from the 1-cell up to the blastocyst stage. This has been demonstrated, for example, by transfer of bovine embryos to sheep or rabbit oviducts (Eyestone et al., 1985; Boland, 1984). These data indicate that the oviduct plays an important role in sustaining embryo development.

If the oviduct creates a unique environment for sustaining embryonic development, then culture of oviductal cells should condition culture medium and improve development of co-cultured embryos. Gandolfi and Moor (1987) cultured one-cell fertilized sheep embryos either in medium alone or co-cultured with a monolayer of fibroblasts or oviductal cells. Embryos cultured either with oviductal cells or with fetal fibroblast cells showed markedly improved rates of development during the first 3 days, compared with those grown in culture medium. When embryos were co-cultured for 6 days, oviduct cells supported blastocyst development in 46 % of embryos, whereas fibroblasts cells produced very few blastocysts (only 5 %). Another striking difference between the ability of oviductal and fibroblast cells to support embryonic development and sustain embryonic viability was evident from the results of embryo transfer experiments (Gandolfi and Moor, 1987). Viability, as indicated by successful pregnancy, of embryos co-cultured with oviductal cells was more than twice that obtained with fibroblasts. Similar experiments with bovine embryos (5- to 8-cell stage) showed that bovine oviductal epithelial cells can support development to the late morula or blastocyst stages, whereas control embryos, in medium only, showed no development beyond the 8- to 16-cell stage (Eyestone et al., 1987). These two experiments clearly suggest that oviductal cells secrete beneficial embryotrophic factor(s) required for normal development. However, these oviductal factors are as yet unidentified.

Uterine Microenvironment

The unique embryotrophic properties of the uterine environment are demonstrated by failure of oviducts, and more precisely, oviductal secretions to provide a favorable environment for embryonic development beyond the early blastocyst stage. Restriction of sheep and pig embryos to the oviduct by ligation of the tubo-uterine junction results in their development to the early blastocyst stage, but no further (Winterberger-Torres, 1956; Murray et al., 1971; Pope and Day, 1972). Furthermore, Orsini and McLaren (1967) reported that the tube-locked embryos not only failed to continue development but underwent degeneration within the oviduct. Termination of embryonic growth, at a stage in development when embryos would normally reside within the uterine lumen, suggests that blastocysts have unique nutrient requirements, essential for continued growth and development, which may only be met by the appropriate milieu of environmental components provided by the uterus. The essentiality of this relationship may be due, in part, to increased complexity of conceptus nutrient and hormonal requirements following blastulation (Biggers and Borland, 1976).

In cattle, uterine responsiveness to circulating ovarian hormones, and in particular P4, results in establishment of a supportive embryotrophic uterine environment. As a consequence of luteal P4 production, glandular epithelium of the uterine endometrium develops morphological characteristics and functions of an active secretory tissue (Skjerven, 1956a; Marinov and Lovell, 1968, Wathes and Wooding, 1980). Progesterone-induced endometrial development and secretion of histotroph appears essential for nourishment of the bovine conceptus

Components of the bovine intrauterine environment have been characterized primarily from analyses of uterine flushings (Roberts and Parker, 1974a,b; Roberts et al., 1976; Bartol et al., 1981), P4-induced uterine fluid (Skjerven, 1956b, Dixon and Gibbons, 1979), and uterine milk obtained from unilaterally pregnant cattle (Bartol et al., 1980). These studies indicate that, during the luteal phase of the estrous cycle or in response to exogenous P4, elevated levels of total amino acids, proteins, fatty acids, lipids, carbohydrates, and certain cations are detected in uterine secretions. Thus during the first 15 to 16 days post estrus, a complex embryotrophic environment is established within the uterine lumen.

The array of polypeptides contributed to the bovine uterine environment specifically by endometrium have been characterized electrophoretically (Bartol et al., 1985). Endometrial explants from pregnant and nonpregnant cattle synthesized a complex array of low and high molecular weight acidic radiolabeled polypeptides. Medium from endometrial explants was enriched in polypeptides in four molecular weight and pI classes: (1) < 14 kDa, > 7.2 pI; (2) 19-24 kDa, 5.4-6.3 pI; (3) 28-31 kDa, 6.9-7.3 pI; (4) > 150 kDa, < 5.1 pI. No qualitative differences were noted in the array of proteins released into medium during the 24 h incubation associated with either pregnancy status (cyclic vs pregnant), or stage of gestation Days 16, 19, 22, 24, and 69. Quantitative changes in endometrial protein synthesis were associated with changing reproductive status as endometrial tissues synthesized more radiolabeled proteins as stage of gestation increased. Bovine serum albumin and Ig heavy and light chain polypeptides, as well as other serum-like proteins (Anderson and Anderson, 1977), were prominent components of culture medium. However, neither BSA nor the Ig polypeptide chains appeared as prominent radiolabeled components of medium, suggesting that such proteins appear in utero, and as components of bovine uterine milk, primarily via transudation from serum and do not represent, directly, major uterine tissue products.

Electrophoretic analysis of uterine flushings collected during the estrous cycle and early pregnancy revealed a complex array of components, several of which appear in the uterine fluids just prior to the phase of rapid conceptus elongation and may be involved in the regulation of this process (Roberts and Parker 1974a,b). Rapid embryonic elongation is associated with the appearance of numerous glycosidases in the uterine fluids (Roberts and Parker, 1974a,b; Roberts et al., 1976). These enzymes may modify glycoproteins and glycolipids on the surface of the endometrium and blastocyst, and in doing so, play an important role in both the induction of embryonic growth and the adhesion phase of implantation (Roberts et al., 1976).

Uterine blood flow varies regularly throughout the estrous cycle in cattle (Ford et al., 1979) and sheep (Greiss and Anderson 1969). Uterine blood flow is greatest at or just prior to estrus and least during the luteal phase of the cycle. These regular patterns of uterine blood flow are associated temporally with the ratio of E2 and P4 in systemic blood (Ford et al., 1979). The greater the ratio of E2 to P4, the greater the quantity of blood flowing through the uterine vascular bed. Exogenous E2, administered on Days 10 or 18 of the estrous cycle, induced marked increases in uterine blood flow and uterine production of PGF in cyclic dairy cows (Knickerbocker et al., 1986).

In addition to steroids, prostaglandins may be involved in altering the rate of uterine blood flow. Prostaglandins may affect changes in blood flow through their effects on arterioles and the capillary bed supply of the uterus and ovary. Infusion of PGE_2 or PGE_2 into the uterine artery caused dilation, whereas PGF induced vasoconstriction of the uterine vasculature (Resnik and Brink, 1978; Still and Greiss, 1978).

One of the earliest physical responses of the maternal unit to the presence of a conceptus in the uterine lumen is an increase in uterine blood flow and/or vascular permeability. In pregnant cattle, a transient two- to three-fold increase in blood flow to the uterine horn containing the conceptus has been observed at the time of maternal recognition of pregnancy between Days 15 and 17 post estrus (Ford et al., 1979). In addition, Amoroso (1952) indicated that before attachment of the bovine conceptus, there is increased edema, vascularity and glandular hypertrophy of the uterine endometrium. The conceptus may be intimately responsible for this pregnancy-induced increase in uterine blood flow, as suggested by the observation that blood flow increased only to the uterine horn containing the conceptus (Ford et al., 1979).

Conceptus production of various agents may be involved in regulating uterine and ovarian blood flow. The local vasodilatory effect of estrogens (Shemesh et al., 1979; Chenault, 1980) and prostaglandins (Shemesh et al., 1979; Lewis et al., 1982) secreted into and sequestered within (Lewis et al., 1982) the uterine lumen by the bovine conceptus may regulate uterine blood flow to the gravid uterus during pregnancy. In addition, prostaglandins may effect changes in permeability of the endometrial vascular bed (Kennedy, 1980) enhancing fluid and electrolyte transport across the uterine epithelia (Biggers et al., 1978). A selective increase in blood flow and permeability of the uterine vasculature system, induced locally by the conceptus, may increase potential for enhancement of histotropic nutrient availability to the developing conceptus and increase exchange of substances between uterine and vascular components important for initiation of luteostasis.

Prostaglandin Dynamics Associated With Lifespan of The Corpus Luteum

Uterine Control of Corpus Luteum Function

Sexually mature bovine females exhibit recurring estrous cycles of approximately 20 to 21 days which are not affected appreciably by season of the year. Essential to recurring estrous cycles is regression of the corpus luteum (CL) which appears to be regulated, to a large extent, by interactions between the ovary and the uterus. During late diestrous-early proestrous phases of the estrous cycle, estrogens produced by the developing, large antral follicle(s) are thought to initiate the process of luteal regression. Exogenous estrogens are known to shorten lifespan of the CL in cattle (Greenstein et al., 1958; Wiltbank, 1966; Eley et al., 1979). Luteolytic activity of follicular estrogen is thought to be mediated through stimulation of uterine prostaglandin $F_{2\alpha}$ (PGF) synthesis and release. Luteolytic doses of E2 were ineffective in hysterectomized (Brunner et al., 1969) and indomethacin (a prostaglandin synthetase inhibitor) treated cyclic cattle (Fairclough et al., 1981).

The luteolytic activity of exogenous PGF in cattle has been documented extensively (Hansel et al., 1973; Hafs et al., 1974; Lauderdale, 1974; Thatcher and Chenault, 1976). For example, elevated levels of PGF in uterine venous drainage (Nancarrow et al., 1973; Shemesh and Hansel, 1975), uterine tissue (Shemesh and Hansel, 1975) and uterine flushings (Bartol et al., 1981a) that occur after Day 15 of the estrous cycle coincide closely with transient decreases in P4 accompanying luteolysis and increases in follicular E2 prior to ovulation in cyclic cattle (Nancarrow et al., 1973; Chenault et al., 1975). Furthermore, Peterson et al. (1975) Kindahl et al. (1976) and Thatcher et al. (1986) reported elevations in peripheral plasma 13,14-dihydro,15-keto PGF (PGFM), the inactive metabolite of PGF, which were temporally associated with CL regression in cyclic cattle. Fairclough et al. (1981) demonstrated that luteolysis can be blocked by passive immunization with PGF antiserum on Day 16 of the estrous cycle. Collectively these observations clearly support the concept that follicular estrogen secretion regulates onset of luteolysis via uterine secretion of PGF.

Regulation of Uterine PGF Production

Although the demise of the CL is uterus and PGF dependent, the interplay is between ovarian E2, P4 and oxytocin (Oxy) with the uterine endometrium to regulate PGF production during the estrous cycle. Estrogens appear to constitute an important stimulatory component regulating uterine PGF production. Synthesis and metabolism of PGF by the uterus in response to E2 administration are documented extensively (Thatcher et al., 1984,1986; Knickerbocker et al., 1986). Exogenous E2, administered on Days 13 or 18 of the estrous cycle, induced marked increases in uterine blood flow, and uterine production and metabolism of PGF in cyclic dairy heifers. Induction of uterine PGF production may be attributed, partially, to E2-dependent increases in activities of cyclooxygenase (Huslig et al., 1979) and phospholipase A_2 (Dey et al., 1982). Calcium is probably involved in activating phospholipase A_2 since this enzyme is Ca^{+2} dependent (Brockerhoff and Jensen, 1974).

Exogenous E2, administered to pregnant and cyclic heifers at Days 19 and 20 of the estrous cycle, resulted in marked differences in uterine responsiveness to E2 challenge due to pregnancy status (Rico et al., 1981; Thatcher et al., 1984). Peripheral PGFM concentrations and the rate of PGFM increase post E2 injection were lower in Day 19 pregnant compared to cyclic cattle, and PGFM concentrations were further decreased in Day 20 pregnant cows. Five of fourteen pregnant cows treated failed to have an E2-induced increase in PGFM. A similar effect of pregnancy, attenuating an Oxy-induced release of uterine PGF on Days 17 to 19 post estrus has been reported (LaFrance and Goff, 1985). These results suggest, that presence of a conceptus supresses uterine release of PGF in response to stimulation by E2 and Oxy. As the conceptus continues to develop and occupies additional surface of the endometrium e.g., Days 18 to 20, uterine PGF production is further diminished.

In addition to estrogen, oxytocin appears to be involved in controlling estrous cyclicity and luteal lifespan in cattle. Oxytocin induces premature regression of the corpus luteum when administered early in the estrous cycle to heifers and this effect was blocked by hysterectomy (Armstrong and Hansel et al., 1959) or indomethacin treatment (Cooke and Homeida, 1983) suggesting involvement of uterine PGF in oxytocin-induced luteal regression. In fact, oxytocin, when administered on Days 1 through 6 (Newcomb et al., 1977) or 17 to 18 of the estrous cycle (Lafrance and Goff, 1985), stimulates uterine production of PGF. The concept that oxytocin plays a role in estrous cyclicity was further supported by the observations that uterine concentrations of the oxytocin receptor increase at luteolysis, to peak at estrus (Roberts et al., 1976; Sheldrick and Flint, 1985), and immunization against oxytocin delays luteolysis in sheep (Sheldrick et al., 1980; Schams et al., 1982) and goats (Cooke and Homeida et al., 1985).

Oxytocin is synthesized in (Wathes and Swan, 1982; Fields et al., 1983; Swan et al., 1984) and secreted by (Flint and Sheldrick, 1982a,b) the corpus luteum, exclusively by the large luteal cells (Rodgers et al., 1983). It appears that oxytocin of luteal (rather than pituitary) origin may principally be involved in the control of luteal function (see Flint et al., 1986).

Oxytocin stimulates uterine PGF production via oxytocin-receptor mediated stimulation of hydrolysis of membrane phosphoinositides to diacylglycerol and inositol phosphates with subsequent release of arachidonic acid from diacylglycerol (Flint et al., 1986; Hixon and Flint, 1987). Liberated arachidonic acid would be expected to lead to increased prostaglandin synthesis. The release of free arachidonic acid is a rate limiting step in the cascade for prostaglandin biosynthesis, and the quantity released may be reflected in the amount of prostaglandin synthesized (Marshall et al., 1987). Arachidonic acid is present in large quantities within the endometrium between Days 10 and 14 of the estrous cycle (Hansel et al., 1975). This increase precedes increases in uterine PGF production observed in cyclic cattle in vivo.

A proposed mechanism for controlling the release of PGF from the uterus of the cyclic sheep, which has been extended to the cow, has been developed by McCracken et al. (1980). According to the model, declining endometrial P4 receptor populations reduce P4 dominance of the uterus during late diestrus and permit E2 from developing follicles to induce oxytocin receptors on the uterine endometrium, making the uterus increasingly responsive to estrogen and oxytocin (Hixon and Flint, 1987). Subsequently, oxytocin released from the neurohypophysis and/or CL binds to newly synthesized endometrial receptors and causes synthesis and pulsatile release of PGF. As a consequence, a series of luteolytic pulses of PGF initiates a rapid decline in plasma P4 and causes regression of the CL (Hixon and Flint, 1987). This pulsatile pattern of PGF secretion in association with CL regression is common to all large domestic species (Thatcher et al., 1986).

During pregnancy, the episodic secretion of PGF is markedly reduced or absent in pregnant cows (see Thatcher et al., 1985). Furthermore, pregnancy attenuates an oxytocin-induced release of uterine PGF in vitro (Gross et al., 1988) and in vivo on Days 17 to 19 post estrus in cattle (Lafrance and Goff, 1985) and Days 13 and 14 of pregnancy in sheep (Fairclough et al., 1984). The mechanism(s) by which the postulated action of oxytocin may be blocked most likely involves the secretion of anti-luteolytic conceptus proteins. An indepth discussion of antiluteolytic/antiPGF effects of conceptus proteins will be presented in a subsequent section of this chapter.

Utero-Ovarian Vasculature

Luteolysis in cattle is thought to occur via a local veno-arterial countercurrent pathway involving veins which drain the uterus and the ipsilateral ovarian artery (Mapletoft et al., 1975; Ginther, 1981; Wolfenson and Thatcher, 1983a). A main uterine branch of the ovarian vein and ipsilateral ovarian artery provide an adequate uterine outlet and ovarian inlet, respectively, for countercurrent exchange of PGF. Support for the concept of local countercurrent exchange of luteolytic PGF is (1) elevated endometrial and uterine vein plasma PGF concentrations that occur between Days 15 and 20 of the estrous cycle (Nancarrow et al., 1973; Shemesh and Hansel, 1975); (2) a preferential transfer of PGF from the uterine vein to ovarian artery follows intra-uterine deposition of PGF (Hixon and Hansel, 1974); (3) elevated E2-induced concentrations of PGF are detected in the uterine branch of the ovarian artery compared to the facial artery in cyclic cattle on Day 18 post estrus (Knickerbocker et al., 1986); and (4) during pregnancy (Days 17 to 20), lower concentrations of PGF were detected in the ovarian artery of pregnant compared to that of cyclic cows (Wolfenson and Thatcher, 1985), which is consistent with previous evidence for a conceptus induced antiluteolytic effect.

Prostaglandin Biosynthesis by Uterine Endometrium

The previous experiments suggest that cyclic cattle enter a luteolytic pathway, whereas pregnant cattle enter a embryotrophic pathway of which luteal maintenance is an essential component. Critical to understanding this process in cattle is whether endometrial production of prostaglandins differs between pregnant and cyclic cattle at the time when the process of pregnancy recognition has been initiated.

De novo synthesis of PGF and PGE from endometrial tissues of cyclic and pregnant cattle has been examined at Day 17 post oestrus (Curl et al., 1983; Gross et al., 1988). Endometrium from cyclic cattle, incubated in medium supplemented with arachidonic acid, produced more PGF and smaller amounts of PGE_2 compared to endometrium from pregnant animals (Curl et al., 1983)

Recently a bilateral perifusion device was developed to allow for discrimination between secretion of prostaglandins towards the luminal and myometrial surfaces of the endometrium (Lacroix and Kann, 1983). This device has been utilized to measure PGF secretion by endometria obtained from cyclic and pregnant cattle on Day 17 post oestrus (Gross et al., 1988a). Regardless of reproductive status, PGF secretion rates were higher from the luminal side than from the myometrial side of the endometrium. However, secretion rates of PGF were lower for endometria of pregnant than those of cyclic cows. Furthermore, PGF secretion by endometria of pregnant cows was not stimulated by oxytocin, whereas copious amounts of PGF were secreted by endometria of cyclic cows in response to oxytocin. Decreased endometrial secretion of PGF during early pregnancy suggests that the CL is maintained due to the inhibition of endometrial prostaglandin synthesis, or due to an inability of the endometrium to respond to stimulators of PGF synthesis such as estrogen and oxytocin.

Since maintenance of the corpus luteum and pregnancy recognition at Day 17 post insemination are associated with alterations in endometrial prostaglandin synthesis, it is important to characterize potential factors which regulate endometrial prostaglandin biosynthesis during this time period. The bovine endometrium contains an inhibitor of prostaglandin biosynthesis (Wlodawer et al., 1976), and recent evidence indicates that reduced secretion of prostaglandin by endometrium of early pregnant cattle is due to increased activity of a endometrial inhibitor of prostaglandin synthesis starting on Days 14 to 17 of gestation (Basu and Kindahl, 1987; Gross et al., 1988b). Gross et al. (1988a) utilized the microsomal fraction of parturient bovine cotyledons as a source of enzymes for prostaglandin synthesis to test the ability of endometrial cytosolic supernatants, harvested from homogenized endometrium from Day 17 cyclic and pregnant cattle, to inhibit prostaglandin synthesis. Cytosolic supernatant from cyclic cows decreased, to a slight extent, the synthesis of PGF by the cotyledonary microsome prostaglandin generating system, whereas preparations from pregnant cows decreased PGF synthesis markedly. The mechanism of inhibition appears to be non-competitive with respect to arachidonic acid. The inhibitor is proteinaceous and can be precipitated by 20 % saturated ammonium sulfate. When resolved by HPLC gel-filtration, the inhibitor activity occurred in two molecular weight regions (20 to 25 and 65 to 70 kDa) and co-eluted with peroxidase activity that was also higher in pregnant endometrium (1988d).

These data clearly indicate that attenuated uterine prostaglandin production during early pregnancy in cattle appears to be associated with, and most likely caused by, induction of an intracellular endometrial inhibitor(s) of prostaglandin synthesis. Bovine conceptuses may exert a local paracrine effect on the endometrium, through secretion of proteins into the uterine lumen, to induce this inhibitor of prostaglandin synthesis.

Role of the Conceptus in Establishment of Pregnancy

Establishment of pregnancy involves interactions between the conceptus and uterus. Conceptuses of domestic animals produce proteins, steroids and prostaglandins during early pregnancy which may be involved in protecting the CL from the luteolytic effects of PGF produced by the uterine endometrium. Maintenance of CL function and continued production of progesterone are essential if pregnancy is to persist in cattle.

The bovine conceptus must be in utero to signal its presence to the maternal system by Days 16-17 of pregnancy (Betteridge et al., 1980). Cows from which conceptuses were removed on Days 17 and 19 had extended interestrous intervals of 25 ± 1 and 26 ± 1 days compared to intervals of 20 ± 1 days when conceptuses were removed on Day 13 (Northey and French, 1980). Also, transfer of embryos to recipient cows on Day 16, but not on Day 17 of the estrous cycle, resulted in normal pregnancies (Betteridge et al., 1980). Furthermore, twice daily intrauterine injections of Day 17 to 18 conceptuses homogenates (Northey and French, 1980) or freeze-killed Day 16 conceptuses (Dalla Porta et al., 1983) to cyclic cows on Days 14 to 18 or 15 to 19 of the estrous cycle, respectively, extended the interestrous interval to 24 days and delayed decline in P4 associated with CL regression. Conversely, intrauterine infusions of Day 12 conceptuses were without effect on CL life-span.

Conceptus Secretory Proteins

Bovine conceptuses (Days 16, 19, 22, 24 and 27) cultured in the presence of radiolabeled amino acids, synthesize and secrete several radiolabeled polypeptides into culture medium. De novo protein synthesis by conceptuses was determined by post-incubation analysis of undialyzable radiolabeled components of conditioned culture medium by two dimensional polyacrylamide gel electrophoresis and fluorography (Masters et al., 1982; Bartol et al., 1985a). The major proteins secreted between Days 16 and 24-27 have molecular weights of 22,000 to 26,000 and pIs of 5.5 to 6.8 (Bartol et al., 1985a). These proteins are referred to as bovine trophoblast protein 1 (bTP-1) complex (Helmer et al., 1987). Other less prominent polypeptides identified included those in the molecular weight and pI range of 40 to 50 kDa and 6.2 to 7.4, respectively. The ability of these early conceptuses to incorporate amino acids and synthesize and release proteins appears to be related to the process of elongation of the trophoblast or their developmental stage.

Patterns of polypeptides synthesized by Day 19 and later stage conceptuses were more complex than those of Day 16 conceptuses and there was a general qualitative and quantitative increase in protein production. Higher molecular weight products including several polypeptides in the 40 to 70 kDa, 7.4 to 5.5 pI range and a large acidic product in the > 400 kDa, < 5.0 pI range were detected. A high molecular weight (> 660 kDa) glycoprotein was identified as the major [^{3}H]glucosamine-labeled product of Day 19 bovine conceptuses. This glycoprotein of high molecular weight was comprised of a least 50 % carbohydrate (Masters et al., 1982).

An array of proteins has been identified as being produced in vitro by sheep conceptuses between Days 13 and 21 of pregnancy (Godkin et al., 1982). One of these, ovine trophoblast protein-1 (oTP-1), has been purified and is a low molecular weight (~ 19 kDa) acidic (~5.5 pI) polypeptide. The oTP-1 is the only detectable protein produced at Day 13 and is produced only up to Day 21 based upon assessment of 2-D PAGE fluorographs. A rabbit antiserum has been prepared to oTP-1, and the following responses involving oTP-1 have been identified: (1) oTP-1 is localized in trophectoderm cells (Godkin et al., 1984a); (2) it accumulates in endometrial epithelium of Day 16 pregnant ewes (Godkin et al., 1984a), (3) receptors for oTP-1 are present on endometrium (Godkin et al., 1984a); and (4) oTP-1 extends the lifespan of the CL (Godkin et al., 1984b).

Antiserum to oTP-1 cross-reacts with two molecular weight components of unfractionated bovine conceptus secretory proteins (Helmer et al., 1987). Furthermore, electrophoretic analysis of radiolabeled conceptus secretory proteins immunoprecipitated with oTP-1 antiserum revealed that peptides present in the precipitate had molecular weights of 22 to 24 kDa (Helmer et al., 1987). These data clearly suggest a potential partial homology between low molecular weight acidic proteins produced by sheep (oTP-1) and cattle (bTP-1) conceptuses during the period of establishment of pregnancy (Helmer et al., 1987). This interspecific conservation in trophoblast proteins, in addition to evidence that luteal maintenance in recipient cows and ewes after interspecific transfer of trophoblastic vesicles (Heyman et al., 1984), argues strongly in favor of a similar mechanism leading to establishment of pregnancy in both sheep and cattle.

Chemical characterization of the anti-oTP-1 immunoprecipitated protein complex indicated that bTP-1 is comprised of seven isomers of N-linked glycoproteins in two size classes, a 22 kDa high-mannose isomer and a 24 kDa complex type isomer (Anthony et al., 1988; Helmer et al., 1988a). Anti-oTP-1 antiserum also recognized a major cell-free translation product of Day 17 bovine conceptus mRNA (Helmer et al., 1987). This translation polypeptide has a molecular weight 4000 to 6000 less (i.e. ~18 kDa) than bTP-1, suggesting some degree of post-translational processing of the initial product, such as glycosylation.

Antiluteolytic-AntiPGF Effects of Bovine Conceptus Secretory Proteins

Experiments have been conducted to test whether bovine conceptus secretory proteins (bCSP) alter luteal lifespan or the pulsatile secretion of uterine PGF (antiluteolytic and antiPGF effects) (Knickerbocker et al., 1986a,b). Conceptus secretory proteins, harvested from medium in which 16- to 18-day-old conceptuses were cultured for 24 h, were injected into the uterine lumen of cyclic cows on Days 15 to 21 post estrus. Cows receiving bCSP had extended luteal function and longer interestrous intervals (30 to 39 days) than cows receiving serum proteins alone (23 to 24 days). Cows receiving conceptus proteins also had lower concentrations of PGF in plasma of the inferior vena cava. In a second experiment, cyclic cows received intrauterine injections of bCSP on Days 15 to 18 post estrus, and on Day 18 cows were injected with E2 to stimulate uterine production of PGF. In three of five bCSP treated cows, E2 failed to stimulate a

PGFM response, whereas, all control serum protein-treated cows had an E_2 induced PGFM response. Overall mean concentrations of PGFM were lower for cows receiving bCSP. These results clearly support the hypothesis that conceptus secretory proteins exert an antiluteolytic-antiPGF effect necessary for maintenance of the CL during early pregnancy.

Recently, bTP-1 has been purified with the aid of HPLC gel filtration techniques (Helmer et al., 1988b). Experiments were conducted to determine whether purified bTP-1 exerts antiluteolytic-antiPGF effects similar to that observed with unfractionated bCSPs. Purified bTP-1, obtained from medium in which 16- to 18-day-old conceptuses were cultured, was infused into the uterine lumen of cyclic cows on Days 15 to 21 post-estrus. Cows receiving bTP-1 had longer interestrous intervals (26.0 ± 1.3 days) than those receiving bovine serum proteins (19.5 ± 1.3 days). In a second experiment, endometrial explants from cyclic cows at Day 17 post estrus were incubated with purified bTP-1 or unfractionated bCSP to determine if conceptus secretory proteins alter endometrial prostaglandin production in vitro (Helmer et al., 1988c). Treatment with bCSP and bTP-1 decreased secretion of PGF by endometrial tissues compared to control tissues treated with serum proteins. However, PGE secretion was greater for bTP-1 than bCSP treated explants. These in vivo and in vitro data suggest that purified bTP-1, a component of bCSP, was effective in extending CL function possibly due to its antiluteolytic-antiPGF and antiluteolytic-luteoprotective effects on endometrium and ovary, respectively.

Available evidence indicates that bovine conceptus secretory proteins, and most likely bTP-1, have a direct effect on the endometrium to elicit their antiluteolytic-antiPGF effects. Basu and Kindahl (1987) recently reported an intracellular endometrial prostaglandin synthesis inhibitor (EPSI) during early pregnancy in cattle. Gross et al. (1988b) confirmed the presence of a prostaglandin inhibitor within the endometrial intracellular cytosol of Day 17 pregnant cattle. Incubation of endometrial explants from Day 17 post estrus cyclic cattle with bCSP (Gross et al., 1988c) or purified bTP-1 (Helmer et al., 1988c) for 24 hours induced a marked increase in EPSI activity and decreased endometrial PGF synthesis. These results suggest that bovine conceptuses exert a local paracrine effect through secretion of bTP-1 and/or other proteins to induce an endometrial intracellular inhibitor of PGF synthesis. If the prostaglandin generating system of the endometrium is inhibited by the conceptus, due to inhibition of key regulatory enzymes such as phospholipase A_2 or cyclo-oxygenase, then the effects of normal endogenous regulators such as estrogen and oxytocin, leading to luteolysis will be blocked However, since the endometrial prostaglandin inhibitor is compartmentalized within the endometrial tissues, it may permit continued active biosynthesis of prostaglandins by the conceptus and their local associated effects necessary for continued embryonic and uterine development.

Recently, the primary amino acid sequence of oTP-1 was partially determined (Imakawa et al., 1987; Stewart et al., 1987). Based on a 70.3 % homology with bovine interferon-α-II (Imakawa et al., 1987) oTP-1 is probably an α-interferon -like molecule. In addition, binding of radiolabeled human α-interferon to membrane receptors from uteri of cyclic ewes is inhibited by purified oTP-1 (Stewart et al., 1987). The inferred primary structure of bTP-1 also indicated that it could be an interferon-like molecule.

Plante et al. (1988) tested possible antiluteolytic effects of E. Coli recombinant bovine interferon-αI type 1 (Capon et al., 1985) in cyclic heifers. Twice-daily intrauterine infusion of interferon from Day 15.5 to 21 post-estrus extended interestrous interval from 22.8 ± 0.8 to 26.8 ± 1.4 days in cyclic cows and extended CL lifspan as determined by serum P4 concentrations. In vitro examination of the antiluteolytic effects of interferon indicated that interferon did not alter the secretion of PGF by endometrial explants of Day 17 cyclic cows and did not induce intracellular EPSI activity in the explants after a 24 h culture period (Plante et al., 1988, unpublished data). Collectively, results indicate that recombinant interferon-

-1 exerts slight antiluteolytic effects, but its mode of action appears to be different from that of bTP-1.

Embryonic Mortality During Preimplantation Development

The classic studies of Hammond (1914, 1921) indicated that the number of potential offspring in domestic animals, as indicated by the number of corpora lutea, was much greater than that realized at term. Since that time, effects of environment, nutrition, genetics, age, disease and endocrine factors have been studied in relation to embryonic death (see Boyd, 1965 and Wrathall, 1971).

In cattle there is a high incidence of embryonic death. Hawk (1979) estimated that rates of fertilization failure were approximately 13 %, whereas rates of embryonic death, as a percentage of cows inseminated, averaged about 25 to 30 %. These are two major causes of reproductive failure of dairy cattle that contribute to an overall failure rate of 40 %.

Timing of Embryo Mortality

One of the most critical periods in the life of the bovine embryo and one of relatively high mortality occurs during the first three weeks of gestation. Rapidly growing embryonic tissues must not only obtain sufficient nutrients and maintain their integrity while free living in the uterine lumen, but at the same time embryos must differentiate in preparation for placental attachment. Studies based on ovum,

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embryo and fetus recoveries at various stages after breeding indicate that early embryonic death between Days 8 and 18 account for 75 to 80 % of all embryonic and fetal mortality in cattle (Ayalon, 1972; Diskin and Sreenan, 1980; Roche et al., 1981).

Cows that fail to become pregnant following three or more consecutive inseminations are classified as repeat breeder cows. Published estimates of the incidence of repeat breeder dairy cows range from 10 to 18 % (Ayalon, 1984). A high incidence of embryonic abnormalities (Linares, 1982) and early embryonic death occurs among repeat breeder dairy cattle with chronic low fertility. Figures ranging from 54.1 % to 65.1 % embryonic death before Day 34 of pregnancy have been reported (Hawk et al., 1955).

In attempts to identify the causes of reproductive failure in repeat breeder cows, a number of investigators have identified several maternal and embryonic factors that may be responsible for causing embryonic mortality in cattle. Intrauterine embryonic mortality is the net effect of numerous factors such as fertilization failure, genetic abnormalities of ova and embryos, altered nutritional and hormonal status of the dam, and alterations in the oviductal and uterine microenvironment.

Genetic Factors Affecting Embryonic Mortality

Among the genetic factors that are known to adversely affect the embryo, chromosomal abnormalities are most prevalent. Chromosomal imbalance (aneuploidy) is a frequent outcome of conception and a cause of embryonic and fetal death. Chromosomal abnormalities are inherited or arise de novo during gametogenesis, fertilization and early cleavage stages (Hamerton, 1971). Cytogenetic analysis of the early bovine embryo indicates that the incidence of early embryonic chromosomal abnormalities which occur during these events range from 0 to 30.7 %, depending on the particular study (Hare et al., 1980; McFeely and Rajakoski, 1968; King et al., 1985).

During gametogenesis, abnormal meiosis can produce gametes with unbalanced chromosomal composition. Such gametes are capable of participating in fertilization and result in embryos with chromosomal abnormalities (Ford, 1972). Chromosomal abnormalities which arise at the time of fertilization are usually those which include whole sets of chromosomes. These result in either polyploid or fertilization-derived chimeric embryos. Abnormalities which might arise during the first few cleavage divisions include (1) non-disjunction leading to the loss of one or more chromosomes from a cell with the possible gain of these chromosomes by its sister cell; (2) failure of mitosis resulting in polyploid nuclei; or (3) the failure of cytokinesis resulting in polynuclear cells.

Very few live-born animals with chromosomal anomalies have been reported, and those observed usually manifest debilitating congenital malformations (Hare, 1979). Hence the genetically abnormal embryo or individual abnormal blastomeres within the embryo fail to survive as development progresses and most are eliminated during the first month of gestation (Williams and Long, 1980; Hare et al., 1980).

Embryo loss in farm animals is usually much higher than can be attributed to chromosomal abnormalities. In fact, genetic abnormalities appear to account for only a small percentage (0 to 4 %) of embryonic mortality in domestic animals (Boyd, 1965; Long and Williams, 1980). Much of the difference between total embryo loss and that arising from the death of embryos with abnormal karyotypes is usually thought to be linked to environmental, nutritional or disease factors.

Uterine Microenvironment and Embryonic Mortality

Embryo transfer studies in the cow have demonstrated the critical importance of the state of the uterine environment for the viability and development of the embryo (Rowson et al., 1972; Sreenan and Beehan, 1974). Embryo transfer between animals that were not in estrus at the same time established that during early pregnancy, embryonic development depends upon an appropriate sequence of changes in uterine secretions to provide histotrophic nutrition to the developing conceptus. Deviation from this relationship due to inappropriate alterations in the composition of uterine secretions may cause abnormal embryo development and lead to increased rates of embryonic death. (Adams, 1971; Wilmut and Sales, 1981).

Ayalon (1978) examined the levels of carbohydrates, total protein and several ions in the oviductal and uterine luminal flushings from fertile and infertile cows during the period 6 to 8 days after insemination to determine whether there were changes in fluid constituents that could be correlated with embryonic mortality in infertile cattle. Carbohydrate concentrations were similar for both types of cattle, whereas total protein levels were consistently higher in uterine flushings from normal cows. Striking differences were found in ion concentrations of both uterine and oviductal fluids. Cows with abnormal embryos had higher concentrations of potassium, zinc, phosphorus and calcium. In a similar study, Lamonthe and Guay (1970) compared the composition of endometrial secretions from normal and repeat breeder cows. Repeat breeders had lower uterine concentrations of sodium, phosphorus, glucose and total protein on Days 5 and 11 post estrus, whereas concentrations of calcium, potassium and magnesium were higher. Decreased total protein and glucose concentrations and alterations in ion composition of uterine and oviductal secretions in subfertile cattle may limit nutrient availability to the developing embryo and alter the pH and osmolarity of the embryo's microenvironment. Since uterine fluid composition in cattle may be controlled by ovarian estrogen and progesterone, as discussed previously, potential alterations in the endocrine status of the repeat breeder cow may account for some of the differences observed in uterine and oviductal fluid composition.

Maternal Hormonal Status and Embryo Mortality

Maternal hormonal imbalance is frequently claimed to be connected with embryonic death. Erb et al. (1976) found higher plasma E2 levels in fertile cows, particularly during the preovulatory period 12 h before estrus. Plasma P4 levels were slightly higher during the luteal phase of the estrous cycle in fertile cows. Compared to fertile animals, infertile cows showed a high incidence of hormonal asynchrony, involving P4, LH, and E2 prior to estrus. Similarly, Maurer and Echternkamp (1982) observed hormonal asynchrony in cows with abnormal embryos, in that, the LH peak prior to ovulation was lower, a longer interval from onset of estrus to the LH peak was detected, and lower P4 concentrations in peripheral blood serum were detected. Hormonal asychrony may produce an undesirable uterine environment for gametes or embryos which result in fertilization failure or embryonic death.

Nutrition and Embryo Mortality

Review of the literature on the effects of nutrition on embryonic survival in sheep and cattle indicate that extremes of under- or over-nutrition during early pregnancy are detrimental to the growth and survival of embryos. For example, Parr and Williams (1982) and Parr et al. (1982) reported that feeding ewes a low-plane of nutrition retarded embryonic development by Day 21 of pregnancy and increased the incidence of fetal mortality. Similarly, in cattle, undernutrition is associated with increased rates of embryonic mortality (Spitzer et al., 1978; Dunn, 1980). Undernutrition may increase embryonic loss by inducing asynchrony of the embryo with its uterine environment, a disturbance in the amino acid and protein composition of the uterine environment, or a reduction in the availibilty of glucose.

Estrogenic forages, in particular red clover, contain a high content of isoflavones, which are converted in the rumen to estrogens, cause reduced fertility in sheep and cattle. Although it is generally regarded that the adverse effect of phyto-estrogens is mediated through reduction in fertilization, there is evidence that prolonged grazing of red clover can lead to cystic endometrial hyperplasia and reduced embryonic survival during the late implantation period of pregnancy (see Robinson, 1983).

Uterine Pathogens and Embryonic Mortality

Uterine infections play an important role in reducing fertility by causing abortion in all domestic species. Although a great number of organisms (bacteria, viruses, protozoa and fungi) are involved in enzootic abortion, only trichomoniasis and campylobacteriosis, brucellosis and infectious bovine rhinotracheitis infection unequivocally are associated with increased rates of embryonic mortality. In natural breeding herds, the pathogenesis and symptoms will largely depend upon the immunological status of the female population. In recent outbreaks with almost no immunity in the female population, pathogens pass from infected males to females and enter the uterus within 5 days after infective copulation (see Wouters, 1986). In the uterus, these organisms can induce endometritis, early and late embryonic death and abortion. Bacterial endotoxins stimulate uterine prostaglandin production (Edqvist, 1988 personal communication) and, as a result, may induce partial or complete luteal regression resulting in abortion in infected pregnant cattle.

Heat Stress Effects on Animal Physiology

On the basis of numerous reviews written over the last 20 years (Bianca, 1965; McDowell, 1966; Thatcher, 1974; Thatcher and Collier, 1982, 1986; Beede and Collier, 1986) and books written by Brody (1945), Hafez (1968), and McDowell (1972), it is obvious that high environmental temperatures, relative humidity and solar radiation reduce lactation, growth and reproductive efficiency of domestic animals. Lactating dairy cattle are extremely sensitive to periods of thermal stress. Basically the problem is that environmental temperature is too high for maintaining normal internal heat production resulting from body metabolism. In an attempt to maintain normal body temperatures, the cow makes adjustments in rate of metabolism by reducing feed intake, milk production, and estrous behavior. When these physiological and behavioral modifications fail to counteract environmental heat load, body temperature of cattle increases and fertility is decreased

Thermoregulation

Cattle belong to the subgroup of vertebrates which are homeothermic and have developed successful strategies to maintain body temperature within a narrow range. These thermoregulatory strategies can be divided into either voluntary (behavioral) or involuntary thermoregulation. A high priority is placed on thermoregulation by the mammalian system, so much so, that the thermoregulatory system calls on and presents challenges to other regulatory systems such as the cardiovascular, respiratory, and endocrine systems.

All homeotherms have a range of temperatures which they can withstand without changes in basal metabolism. This range, called the zone of thermal neutrality, is bounded by upper and lower critical temperatures (NRC, 1981). Within the zone, adjustments such as vascular distribution, pelage, or behavior are used successfully to maintain homeothermy. As the critical temperature is exceeded, physiological and metabolic adjustments are needed in order to dissipate heat and maintain body temperature. These adjustments are increasingly deleterious to productive functions of domestic animals such as lactation, growth, and reproduction.

Heat Loss

Animals exchange heat with their environment by means of conduction, convection, radiation and evaporation. The first three are classified as sensible heat loss mechanisms which require a thermal gradient to be effective. The fourth route of heat loss, evaporation, is termed insensible heat loss and does not require a thermal gradient to operate.

Conduction involves the passage of thermal energy from molecule to molecule according to the second law of thermodynamics. Convection is the transfer of thermal energy by circulation of a fluid or gas which is non-uniform in temperature. Thus blood flow is a major avenue of convective heat transfer to the body periphery or skin surface. Radiation is the passage of thermal energy through space without heating the space through which it passes. Evaporative heat loss involves a reduction in thermal energy utilized when water is changed to the gaseous state.

Thus in the thermoneutral zone, sensible heat loss is the major avenue of heat dissipation, and metabolism proceeds independently of ambient temperature. At the upper critical temperature, sensible heat loss is maximal. When the thermoneutral zone is exceeded, the animal must rely on evaporative heat loss and reductions in heat of metabolism to maintain homeothermy.

Cardiovascular Adjustments to Heat Stress

An early response to rising ambient temperature is an adjustment in the cardiovascular system. This response occurs within the thermoneutral zone before upper critical ambient temperatures are reached (Horvath and Rowell, 1964). Adjustments include an increase in cardiac output and blood volume (Hertzman, 1959) and peripheral vasodilation. Control of blood flow to the skin depends on tone and resistance of the capillary bed. Increases in ambient temperature result in vasodilation of skin vasculature and subsequent increases in blood flow.

Both sensible and insensible heat loss are increased by shifts in blood distribution to the skin. Sensible heat loss requires a gradient for heat dissipation. Thus, by vasodilation of peripheral blood vessels, a greater amount of blood and therefore heat is brought to the body surface for dissipation by radiation, conduction, or convection. As long as the temperature gradient is maintained the animal can rely on sensible heat loss as the primary mode of heat dissipation. However, as ambient temperature and/or heat load increases, the gradient disappears and the animal must utilize evaporative heat loss as the primary mode of heat dissipation.

Physiological Fluid Adjustments to Heat Stress

Normally, increases in evaporative heat loss require an expansion of plasma volume to move more water to the body surface for evaporation. This is reflected in lower plasma protein concentrations and hematocrit. Water requirements for expansion of plasma volume, to increase evaporative heat loss, are met by increasing fecal and renal water retention and activation of the thirst drive which increases water intake. McDowell (1972) illustrated how dramatically total body water balance was affected during thermal stress. In climate chambers (18 vs 30 C) drinking water consumption of lactating cows increased 29 % at the warmer temperature; fecal water loss declined 33 %, but loss via urine, skin surface and respiratory evaporation increased 15, 59 and 50 %, respectively. Comparable results were noted for nonlactating cattle, though water loss via sweating was considerably greater (176 % increase at 30 compared to 20 C, McDowell and Weldy, 1967).

Respiratory Adjustments to Heat Stress

When ambient temperatures and solar heat load increase, there is a subsequent increase in the respiratory frequency of cattle (Thompson, 1973). Respiration rates increase as much as 30 to 300 %, depending on the degree of thermal stress to which cattle are exposed (Roman-Ponce et al., 1977). This increase in respiratory rate increases evaporative heat loss from the upper respiratory passages.

Panting is not as effective as sweating in increasing evaporative heat loss (McLean and Calvert, 1972; Macfarlane, 1968) and tends to alter alveolar ventilation, which subsequently alters blood CO_2, O_2, and pH In severe heat, when body temperature continues to rise, panting is replaced by second phase breathing, characterized by low respiratory frequency and large tidal volume (Bianca, 1968). During this phase, alveolar respiration increases dramatically which results in development of respiratory alkalosis (Bianca and Findlay, 1962). Respiratory alkalosis has negative effects on animal performance, especially uterine and umbilical blood flow during pregnancy (Oakes et al., 1976).

Nutritional Adjustments to Heat Stress

Under thermoneutral conditions, feed intake and metabolism proceed independently of ambient temperature. At higher temperatures both feed intake and basal metabolism are reduced. In lactating dairy and feedlot cattle, dry matter intake begins to decline at mean daily environmental temperatures of 25 to 27 C (Beede and Collier, 1986). This reduction in feed intake, resulting in a reduced flow of essential nutrients and metabolizable energy from the digestive tract, is in large part responsible for reduced performance of heat stressed animals. Depression of voluntary feed intake by hyperthermia is believed to be due to several factors including a direct negative effect of temperature on the appetite center in the hypothalamus (Baile and Forbes, 1974). A second cause is reduced gut motility, rumination, and rate of passage of ingesta (Robinson and Klamm, 1953, Warren et al., 1974) which in turn leads to increased gut fill and probably depressed appetite. Reducing dry matter intake in response to thermal stress, reduces heat generated during the processes of ruminal fermentation and body metabolism, and therefore, aides in maintaining normal body temperatures during periods of thermal stress.

Water unequivocally is one of the most important nutrients for heat-stressed animals. Water intake of ruminants is dramatically increased during thermal stress (McDowell, 1972; Ingram and Mount, 1975, Roman-Ponce et al., 1977, 1981). Consumed water substantially cools rumen temperature, thus reducing heat load (Bianca, 1964) and serves as the primary vehicle for heat transfer and dissipation through sweating and panting.

Endocrine Adjustments to Heat Stress

A complex neuroendocrine response is an integral part of the overall temperature regulating mechanism which permits homeotherms to maintain constant body temperature in the face of acutely varying environmental and body conditions (Chowers et al., 1976). Monoamines such as dopamine, epinephrine, norepinephrine, and serotonin are implicated in the molecular control of neurotransmission within the thermoregulatory center of the hypothalamus as well as having peripheral effects on blood flow and sweating rate (Alaveras and

Johnson, 1973). Plasma epinephrine and norepinephrine are elevated following both acute and chronic heat stress in cattle (Alvarez and Johnson, 1973; Robertshaw and Whittow, 1967). This is likely related to increased sweat gland activity induced by catecholamines (Joshi et al., 1968, Allen and Bligh, 1969). Sweat glands of cattle are not innervated directly (Jenkinson et al., 1966), but are regulated by adrenergic hormones synthesized and secreted by the adrenal medulla (Joshi et al., 1968; Allen and Bligh, 1969).

Anterior pituitary function is altered by environmental heating. Primary effects of increased heat load on pituitary function are related to those hormones involved in basal metabolism such thyroid stimulating hormone (TSH), growth hormone (GH), prolactin (PRL), and adrenocorticotropic hormone (ACTH).

As cattle adapt to chronic thermal stress their basal metabolic rate decreases while water and electrolyte turnover increases (Johnson et al., 1967; McDowell et al., 1968; Collier et al., 1982a). These adaptations are reflected in lower concentrations of metabolic hormones. Exposure to heat and subsequent increases in body temperature reduce thyroxine secretion by the thyroid gland in cattle (Bianca, 1965; Collier et al., 1982) and sheep (Brooks et al., 1962). Reduced thyroid function is preceded by a reduction in the secretion of TSH by the pituitary (Anderson, 1964; Krulich et al., 1976). The decline in thyroid activity reduces metabolic rate of the animal and thereby reduces metabolic heat load. Heat-stressed cattle have a reduced plasma GH concentration (Mitra et al., 1972; Mitra and Johnson, 1972). Since GH is involved in control of basal metabolism, a reduction in its secretion would lower metabolic heat production. Injection of GH (200 to 300 mg per cow, iv) has been reported to greatly increase heat production (Yousef and Johnson, 1966).

Acute rapid environmental changes are associated with a sudden rise in plasma glucocorticoid (GC) concentrations when cattle are maintained at elevated environmental temperatures for periods of several days, an initial rise in plasma GC concentrations is detected, followed within a few hours by a return to pretreatment levels, and eventually GC concentrations are depressed in these animals (Alvarez and Johnson, 1973; Bianca, 1965; Christian and Johnson, 1972). Reasons for differing responses to acute and chronic stress are unknown but may be related to altered adrenal metabolism. Cattle which have acclimatized to warm summer conditions or chronically thermal-stressed have reduced GC release in response to an injection of ACTH (Shayanfar et al., 1975; Roman-Ponce et al., 1981; Gwazdauskas et al., 1981). However these animals had much higher P4 release to ACTH than shade protected controls (Roman-Ponce et al., 1981). Since P4 is a precursor of cortisol, the lower GC and higher P4 concentrations in heat-stressed animals may be related to reduced conversion of P4 to cortisol.

A number of acute hormonal responses to thermal stress are associated with altered water and electrolyte turnover. This is a reasonable assumption since evaporative water loss is the major route of heat exchange when ambient temperature approaches body temperature (Collier et al., 1982). Increases in antidiuretic hormone (ADH) concentrations (El-Nouty et al., 1980) are clearly associated with the need to conserve water and increase water intake as water losses in the respiratory tract and skin increase. Increased water turnover requires associated increases in electrolyte turnover to move water through various fluid pools to the evaporative surfaces. A major difference exists between ruminants and nonruminants with respect to location of sodium (Na^+) and potassium (K^+) loss during thermal stress in that nonruminants produce sweat high in Na^+ and low in K^+ concentrations (Lipsett et al., 1961), whereas ruminants produce sweat that is high in K^+ and low in Na^+ concentrations (Beede and Collier, 1986). Accordingly, thermal stress imposes a need to conserve Na^+ in nonruminants and K^+ in ruminants. Aldosterone secreted from the adrenal cortex causes resorption of Na^+ and, concomitantly, a large flux of water at the kidney. Acute heat stress results in increasing concentrations of aldosterone in nonruminants

(Lipsett et al., 1961) and decreasing concentrations during chronic thermal stress in ruminants (Collier et al., 1982).

Behavioral Thermoregulation

Environmental conditions influence animal behavior. These alterations in behavior have physiological significance since they are directed at attempting to thermoregulate body temperature. Cattle exposed to heat frequently sprinkle water over their bodies (Ragsdale et al., 1951), seek shade (Seath and Miller, 1946) and shift grazing or feeding to night-time hours (Payne et al., 1951). A widely recognized effect of heat stress on farm animals is a decreased libido in bulls and decreased expression of estrous behavior in cows.

Heat Shock Response of Eukaryotic Cells

Heat Shock and Cellular Protein Synthesis

Exposure of cells from a wide variety of organisms to an increase in temperature results in decreased normal protein synthesis and the enhanced synthesis of several proteins, which have been referred to as heat shock proteins (hsps). This response has been observed in every species examined and is not specific for a particular tissue as the overwhelming majority of cells respond to heat in a similar fashion. For example in the rat, tissue explants from the heart, lung, thymus, kidney, brain, liver, and adrenal glands produce a similar spectrum of proteins (White and Currie, 1982). Heat shock proteins are thought to protect the cell against the immediate heat shock and to confer thermotolerance against a subsequent heat shock or stress situation so that normal cellular activities are optimally restored after the stress period.

Development and differentiation have important effects on the responsiveness of cells to heat shock. Early embryos are incapable of producing hsps in fruit flies, sea urchins, frogs, mice, rats, and rabbits, species of such evolutionary divergence as to suggest a universal phenomenon. This inability to respond to heat shock begins during oocyte development or at the time of fertilization. In Drosophila, competence to produce hsps is achieved at the time of cellular blastoderm formation (Graziosi et al., 1980); in the sea urchin, at the hatched blastula stage (Roccheri et al., 1981); in the frog, at the late blastula/early gastrula stage (Bienz, 1984); and in mammalian embryos, at the morula/blastocyst stage (Heikkila et al., 1984a).

Heat Shock Proteins

Heat-shock proteins are defined generally as those proteins whose synthesis is sharply and dramatically induced at high temperatures. Although the exact number of proteins induced varies in different organisms, hsps are all acidic proteins with pI between 5.0 and 6.5 and fall into two size classes, large hsps with molecular weights of 68 to 110 kDa and small hsps with molecular weights of 15 to 30 kDa.

Heat shock protein 70 is most highly conserved of the hsps and, as such, has received the greatest attention. The complete amino acid sequence of hsp70 from four eukaryotic species has been determined (Ingolia et al., 1980) and is highly conserved among species. For example the human protein is 73 % homologous to the Drosophila protein and 50 % homologous to the E. Coli DNA product. With few exceptions hsp70 is a constitutive protein of eucaryotic cells, part of it being tightly associated with the microtubular and intermediate filament cytoskeletal systems and with the plasma membrane (Weatherbee et al., 1980; Biessmann et al., 1982; Wang et al., 1981). During heat stress it accumulates in the nucleus (Vincent and Tanguay, 1979; Hover et al., 1983).

Heat shock protein 83 is the second-most highly conserved heat-shock protein. All eukaryotic cells examined produce a prominant hsp in the range of 83 to 90 kDa. Sequence data are not yet available, but antibodies for chicken hsp89 cross-react with human, mouse, frog and Drosophila proteins (Schlesinger et al., 1982). To date, the most interesting property for hsp83 is its transient association with steroid hormone receptor complexes. Monoclonal antibodies prepared against the 8S P4 receptor revealed that hsp90 is a major component of this complex (Schuh et al., 1985). This association is, however, transient. When the receptor is converted to the 4S form, it loses its association with hsp90, binds hormone more tightly, and acquires the ability to bind DNA. Associations of hsp90 with a glucocorticoid receptor (Sanches and Toft, 1985), and a membrane bound ATPase (Burdon and Cutmore, 1982) have also been described. These findings suggest that this hsp may serve to shuttle important membrane proteins around the cell, holding them in an inactive form as it does so (Lindquist, 1986).

Mammalian cells produce hsps of 100 and 110 kDa which do not appear to have counterparts in Drosophila. Immunological localizations of the 100 kDa protein demonstrate its presence on the Golgi (Lin et al., 1982) whereas antibodies against the 110 kDa protein reveal localization in or around nucleoli (Subjeck et al., 1983). Both the 100 and 110 kDa proteins are constituents of normal cells and are glucose regulated (Kasambalides and Lankas, 1985).

All organisms produce one or more small hsps. A general property of small hsps is their massive, but reversible binding to the nuclear skeleton during heat stress (Arrigo et al., 1980; Silver et al., 1983).

Regulation of hsp Synthesis

Control of hsp synthesis involves activation of corresponding hsp genes and, in many organisms, a highly preferential translation of heat stress-specific mRNAs. Gene expression in heat stressed cells is associated with a 10- to 1000-fold increase in the synthesis of cytoplasmic mRNA species coding for hsps (see Nover, 1983). This reprogramming of gene expression in heat stressed cells is tightly coupled to a striking shift in the translation specificity of the ribosomal apparatus in heat-shocked cells greatly favoring the translation of hsp mRNAs. Most pre-heat-shock mRNA species are not translated during heat shock but remain intact in polysomal complexes and can be reactivated and resume normal protein synthesis after shift down to normal growth temperatures (see Nover, 1983).

Physiological Function of hsps

Heat-shock proteins are thought to protect the cell against stressful environmental conditions, such as heat shock, by protecting and/or stabilizing heat sensitive intracellular structures. Important arguments in favor of a physiological function of hsps stems from studies on their intracellular localization. Binding of hsps to distinct subcellular compartments is highly specific with respect to the protein species but frequently also to the temperature conditions. Heat shock proteins were characterized as components of the plasma membrane, golgi apparatus, and the nucleolus (see Nover et al., 1983). In many organisms they are tightly coupled to chromatin or to the nuclear skeleton during heat stress, but not in the recovery period (Arrigo et al., 1980, Vincent and Tanguay, 1982; Key et al., 1982). Moreover, hsp70 is an integral component of the cytoskeleton, which may be a primary target of heat shock treatments

(Falkner et al., 1981). Thus, cellular membrane and the skeletal systems of the nucleus and cytoplasm evidently constitute central sites of hsp action during periods of thermal stress.

Cell cycle arrest is an important part of the heat shock response in all systems so far investigated (Rao and Engelberg, 1965, 1966; Scharf and Nover, 1982); because of its readily reversible characteristics, brief heat treatments have been used for cell cycle synchronization (Kramhoft and Zeuthen, 1971; Zeuthen, 1971) in many cell culture experiments. In tomato cell cultures, the onset of the heat stress period is accompanied by a rapid decline of the mitotic index from 7 to 10 % of exponentially growing, asynchronous cultures to less than 1.5 %. Following restoration of control temperatures, it takes about three hours to normalize the mitotic activity. A wave of mitosis in the recovery period with maximum mitotic index values of 15 to 20 % indicated a synchronizing effect of hyperthermic treatment (Scharf and Nover, 1982)

In eukaryotic cells, heat stress may result in rapid toxicity at temperatures a few degrees above the normal physiological range (Bowler et al., 1973; Hahn, 1982). Sublethal temperature shock leads to an array of cellular changes such as inhibition of energy metabolism (Sinensky et al., 1979), damaged DNA (Warters and Henle, 1982) and chromosomal abberations (Dewey et al., 1971). Heat stress causes major changes in mitochondrial function, including loss of respiratory control and uncoupling of phosphorylation from electron transport (Christiansen and Kvamme, 1969), changes which may be related to alterations in heat-stressed mitochondrial membrane structure (Lepock, 1982).

Heat Shock and Cellular Ultrastructure

Using electron microscopy and immunological methods Welch and Suhan (1985) have characterized a number of morphological changes that occur in the cytoplasm and nucleus of rat embryo fibroblast cells after heat shock treatment. Heat shock resulted in disruption and fragmentation of the golgi complex and structural alterations within mitochondria. Additionally a number of striking alterations were observed within the nuclei of heat-treated cells. Nucleoli appeared less condensed, showed signs of unraveling and there appeared to be a general collapse of the nuclear matrix.

In most eucaryotic cells, cytoplasmic cytoskeletal structural proteins are organized in three-dimensional networks of filaments and cross-linked microtrabeculae (see Pallas and Solomon 1982; Weber and Glenney, 1982). This continuous skeletal system stabilizes the outer membrane and the highly organized cytoplasmic ground substance and also extends into the nuclear compartment. During heat shock there is a collapse of the cytoskeleton on the nucleus (Falkner et al., 1981; Bissemann et al., 1982; Welch and Suhan 1985).

The primary cellular site for the action of heat damage on tissues is membranes (Bowler et al., 1973; Hahn, 1982). Exposure and subsequent adaptation of tissues to high ambient temperature are accompanied by alterations in membrane lipid composition (Anderson et al., 1981; Anderson & Parker, 1982), increased membrane fluidity, (Quinn, 1981; Calderwood et al., 1987) and increased membrane leakiness for various low molecular weight compounds such as electrolytes, vitamins and amino acids (Cann, 1968; Reeves, 1972; Wu and Wallner, 1983). Heat-induced alterations in membrane composition may affect many membrane functions which require an optimal lipid environment for activity. For example, Na^+-K^+ pump activity, which is dependent on membrane fluidity, is increased at elevated incubation temperatures (Stevenson et al., 1983; Bates et al., 1985) possibly due to increased Na^+-K^+ ATPase enzyme activity as reported in heat-shocked Chinese hamster ovary (CHO) cells (Anderson and Hahn 1985).

A comparison of the heat responses of the ATPase enzyme with responses of other proteins located in the plasma membrane reveals a differential sensitivity to hyperthermia. For example, the insulin receptor of HA-1 cells is markedly more sensitive to heat than the ATPase; all of its capacity to bind insulin is lost after 40 min at 45 C. The effect of heat is to reduce the number of competent receptor proteins rather than to alter binding affinity (Calderwood and Hahn, 1983). In contrast, the receptor for epidermal growth factor in rat-1 cells is more heat resistant than the insulin receptor, and the heat-induced effect is to alter binding affinity, not receptor number (Magun and Fennie et al., 1981).

Heat stress has been reported to stimulate membrane phospholipase C activity and phosphoinositide (PPI) turnover (Calderwood et al., 1987) as well as activating cAMP production (Calderwood et al., 1985). A number of schemes have been suggested for involvement of PPI breakdown in thermal cellular toxicity (see Calderwood et al., 1987). The breakdown of PPI to products such as inositol-triphosphate (IP3) and diacylglycerol (DAG) may act as intracellular second messengers leading to the mobilization of intracellular Ca^{+2}. Stevenson et al. (1986) have shown that heat shock increases Ca^{+2} in CHO cells, an increase which seems to be due to both mobilization from internal stores and increased gating across the plasma membrane. Increased Ca^{+2} was accompanied by rises in DAG and IP3 (Stevenson et al., 1986) Heat shock induced activation of phospholipases and elevation of cell Ca^{+2} may be part of a common pathway leading to cell death which appears in a range of cellular trauma and toxic conditions (Alvedano and Bazan, 1975; Johnson et al., 1983; Wielock et al., 1984).

Heat Stress Effects on Reproductive Processes of Female Cattle

Conception Rates

In subtropical and tropical areas of the world, a predominant environmental effect on fertility is the suppression of pregnancy rates during stressful summer months of the year. Conception rates to artificial insemination may range from 55 % during months of low temperature and humidity to only 10 % during months of high temperature and humidity (Ingraham et al., 1974; Badinga et al., 1985). A considerable number of studies have quantified various climatic measurements and evaluated their associations with conception rate in cattle (Stott and Williams, 1962; Ingraham et al., 1973; Gwazdauskas et al., 1975; Badinga et al., 1985). In a study by Gwazdauskas et al. (1975) and a follow up study by Badinga et al. (1985) various climatological measurements, such as maximum, minimum and average daily air temperature, average daily rainfall, average wind movement, average daily solar radiation, and average daily minimum and maximum relative humidity, were evaluated for effects on fertility of a commercial dairy herd in Florida. Overall, heifers had consistently higher conception rates than lactating cows (47 vs 32 %, respectively) and suffered only slight depression of conception rates during months of thermal stress. Conception rates of cows decreased much more during the summer months of June to August and did not recover until November, suggesting carry-over effects during the months of September and October. Specific climatic measurements were related quantitatively to fertility. Maximum environmental temperature on the day after insemination was the most potent environmental measurement negatively associated with fertility. A clear difference in conception rates was detected between lactating dairy cows and heifers as maximum environmental temperature increased the day after insemination. Conception rates decreased from 52 to 32 % in lactating cows as maximum air temperature increased from 23.9 C to 32.2 C, and the decrease was accelerated when maximum environmental temperatures exceeded 30 C. In contrast, conception rates increased slightly for heifers. This decrease in conception rates of lactating cows is probably due to an inability to maintain a normal body temperature under heat stress conditions because of the high rate of internal heat production associated with the production of milk. Conversely, non-lactating heifers are most likely to have a higher upper critical environmental temperature beyond which body temperature is elevated, but not to the point where fertility is diminished.

There is considerable evidence that thermal stress can influence a number of reproductive processes in female dairy cattle. Thermal stress may act by influencing sexual behavior, the quality of ova ovulated, viability of embryos, as well as eliciting specific responses by the dam that subsequently affect oviductal and uterine environments which may directly influence embryonic viability and growth rates.

Estrous Behavior

Herd reproductive inefficiency results from two main problems: (1) an absence of expressed estrus or insufficient detection of estrus; and (2) infertile services, of which embryonic mortality in dairy cattle is estimated to be approximately 15 % (Hawk, 1979). During hot seasonal periods, the magnitude of these problems is amplified considerably. In heat-stressed cattle, there is a reduction in the length of estrus from 18 to approximately 10 hours and a lower intensity of estrous behavior (Gwazdauskas et al., 1981; see Thatcher and Collier, 1982). Reduced sexual activity lowers metabolic heat production and reduces heat load that must be dissipated by the cow. Such behavioral alterations would contribute to a reduced success of heat detection for artificial insemination programs. For example, in a large commercial dairy herd, the percentage of potential heats not detected increased from a yearly mean of 66 % to over 80 % during the hot summer months of July and August (Thatcher et al., 1984). This, coupled with lower conception rates to detected heats, accounts for a tremendous amount of herd infertility during the summer period of thermal stress.

Hormonal Alterations During the Estrous Cycle

When examining the effects of thermal stress on the array of hormones controlling reproductive processes, it is important to realize that both acute and chronic thermal stress result in altered secretion rates of metabolic hormones, in order to accommodate altered physiological processes associated with dissipation of heat, water and electrolyte turnover, and altered metabolism, as discussed previously. These changes may, both directly and indirectly, alter control and secretion of reproductive hormones.

Increases in uterine PGF secretion in response to heat stress have been reported. Thermal stress between Days 8 and 16 after insemination increased PGFM in the peripheral circulation of gilts. This increase in secretion of PGF may compromise luteal function as indicated by reduced plasma concentrations of progesterone during Days 13 through 19 (Hoagland & Wettemann, 1984; Wettemann et al., 1984). In dairy cattle, prepartum heat stress increases peripheral concentrations of PGFM and reduces CL diameter post partum (Lewis et al., 1984). Decreased concentrations of progesterone in plasma of gilts and CL size in cattle may be due to heat-induced partial regression of the CL in stressed animals, which may be attributed to greater uterine secretion of PGF (reflected by greater concentrations of systemic PGFM) in heat stressed than in thermoneutral animals. Since maintenance of luteal function in cattle is associated with decreased endometrial PGF secretion, it is likely that increases in endometrial prostaglandin secretion rate in response to heat stress may initiate partial or complete luteal regression and contribute to pregnancy failure.

With regard to reproductive hormones controlling the estrous cycle, Roman-Ponce et al. (1981) monitored daily changes in plasma hormonal concentrations of E2, LH and P4 throughout the estrous cycle of cows maintained in a shade management system versus cows managed under a stressful no-shade management system. In both groups of cows, a transitory fall in P4 concentrations associated with corpus luteum regression and a pro-estrous rise in E2 were sufficient to induce a preovulatory surge of LH and estrus followed by ovulation. In cows managed with no shade, slight elevations were detected in P4 during the luteal phase, whereas the pro-estrous rise in E2 concentrations was slightly lower. Similarly, a smaller pro-estrous rise of E2 in heat stressed heifers (32 vs 21 C) was detected when blood samples were collected at 6 h intervals in an environmental chamber (Gwazdauskas et al., 1981). Madan and Johnson (1973) reported that mean baseline plasma LH values as well as the preovulatory peak of plasma LH are reduced in heifers maintained at 35 C, 55 % relative humidity (RH), compared to LH levels in heifers maintained at 18.2 C and 55 % RH. Miller and Alliston (1974) also observed decreased preovulatory and basal levels of LH in thermal-stressed heifers.

The biological significance of these differences in preovulatory LH, luteal P4 and pro-estrous E2/P4 ratios may be related to the quality of the developing pre-ovulatory follicle, the intensity of estrous behavior, and the subsequent microenvironment of the uterus and oviduct. It is interesting to note that alterations in the E2/P4 ratio, similar to that observed during heat stress have been reported to occur in subfertile and repeat-breeder cattle which experience early embryonic mortality (Erb et al., 1976; Maurer and Echternkamp, 1982). Alterations in maternal E2 and P4 concentrations may alter transport of ova and embryos (Chang, 1966) or sperm (Hawk, 1975) through the reproductive tract. In addition altered ratios of E2/P4 may have marked effects on uterine blood flow and alter oviductal and uterine secretions, creating an unfavorable environment for successful early embryonic development.

Alterations in Oviductal and Uterine Environments

Changes in climatic measurements represent alterations in the animal's microenvironment that are related to conception rates. Similar associations can be detected within the potential embryo's microenvironment, e.g. the oviducts and uterus. Uterine temperatures recorded on the day of insemination and the day after insemination were related negatively to conception rate (Gwazdauskas et al., 1973). An increase of 0.5 C above the mean uterine temperatures (38.6 C) decreased conception rates by 12.8 %.

Since elevated environmental and uterine temperatures near the time of fertilization are related to conception rates, the factors controlling oviductal and uterine temperature are important. The greatest rate of uterine blood flow during the estrous cycle occurs during the periestrous period in association with high ratios of E2/P4 concentrations, as discussed previously (Ford et al., 1979). In ovariectomized dairy cows exposed to heat stress during the summer without shade protection, the increase in uterine blood flow induced by E2 was less than that of the same cows treated with E2 but exposed to an effective shade structure (Roman-Ponce et al., 1978a). Similarly, in ovariectomized ewes, uterine blood flow increased less after estradiol injection in ewes exposed to 32 C than in ewes maintained at 21 C (Roman-Ponce et al., 1978b). Thus, during the animal's thermoregulatory response to thermal stress, there is a decreased rate of blood perfusion to the uterus after injection of E2. Such a thermal stress-induced reduction in uterine blood flow would preferentially elevate uterine and oviductal temperatures and probably affect the availability of oxygen, water, electrolytes, nutrients and hormones to the uterus. This may be critical during periods of elevated uterine blood flow that normally occur during the periestrous period or early pregnancy at 14 to 16 days post estrus (Ford et al., 1979). Such induced inhibitory responses, as a consequence of thermal stress, would have a high probability of increasing rates of embryonic death during early pregnancy

A decrease in blood flow to the uterus and oviducts may alter the potential embryo's micro-environment within the oviduct and uterine lumen. For example, Thorne et al. (1980) collected oviductal fluid from pregnant rabbits exposed to heat stress (33.2 C) during the first 5 days of pregnancy. Heat stress induced a marked reduction in oviductal fluid volume and total protein content. A similar decrease in oviductal fluid volume in response to heat stress has been reported in sheep (Rowan, 1965). Decreased oviductal fluid volume in heat-stressed animals may be related to heat-induced alterations in the plasma E2, P4 and catecholamines which may result in decreased activity of oviductal secretory epithelial cells and/or a decrease in transudation from the vascular system due to shunting of blood away from non-vital organs, such as the uterus, to cutaneous vessels to increase body cooling. Injection of E2 has been shown to increase (Clewe and Mastroianni, 1960) and P4 to decrease (Bishop, 1956) the volume of oviductal fluid secreted by ovariectomized rabbits. Since the early cleavage stage embryo, while still in the oviduct, is extremely sensitive to adverse effects of maternal hyperthermia, these heat-induced alterations in potential embryo's oviductal microenvironment may adversely affect embryonic viability.

Early Embryo Development

Specific periods during which the early bovine embryo is sensitive to heat stress have been documented. Several studies indicate that fertilization of the oocyte occurs normally in summer heat-stressed cows (Monty and Racowsky, 1987; Gordon et al., 1987). Embryo recovery at Day 7 post-insemination revealed a high incidence of cleavage stage embryos in both heat-stressed and shade-protected cows. Subsequent to fertilization, embryos are extremely sensitive to maternal hyperthermia. Dunlap and Vincent (1971) exposed beef heifers to temperatures of either 21.1 or 32.2 C for 72 hours following insemination. None of the heifers exposed to heat stress maintained pregnancy compared with 12 of 25 heifers maintained at 21.1 C.

Exposure of superovulated dairy cows to summer heat stress conditions (Gordon et al., 1987; Monty and Racowsky, 1987) during the first 7 days of pregnancy, resulted in decreased quality (Gordon et al., 1987) and viability of morulae and blastocysts (Monty and Racowsky, 1987) recovered from heat-stressed cows compared to those recovered from cows flushed during the winter/early spring cool season. Collectively, these data indicate that the early bovine embryo (Days 1 to 7 post insemination) is sensitive to harmful effects of maternal hyperthermia.

Biggers et al. (1987) evaluated whether elevated ambient temperature between Days 8 and 16 post insemination altered conceptus development in beef cattle. Multiparous cows were acclimated to metabolism stalls in a temperature-controlled building and on Day 7 post insemination exposed to either a control (C: 22 C, 25 % RH), mild heat stress (MHS; 37 C, 27 % RH) or severe heat stress (SHS; 37 C, 40 % RH) treatment until Day 16 when cattle were slaughtered and embryos recovered. Although heat stress did not significantly alter pregnancy rates, there was a trend towards greater embryonic mortality in heat-stressed cows (C: 83%; MHS: 64%; SHS: 50%). Conceptus weights were decreased in cows exposed to MHS and SHS compared with controls. Results indicate that heat stress during the period of blastocyst development, elongation and pregnancy recognition reduces conceptus weights, and this may lead to increases in embryonic mortality. However, thermal sensitivity of the bovine embryo after Day 7 of development does not appear to be as great as during the earlier period of development before Day 7.

The period of embryonic sensitivity to heat stress has been studied more extensively in sheep. Ewes subjected to high ambient temperatures (32.2 C) at the time of mating had lower fertilization rates and increased incidence of early embryonic death (Dutt et al., 1959). In a similar study, ewes exposed to 32.2 C either at the time of breeding (0), or 1, 3, 5 or 8 days after breeding had similar rates of fertilization, but increased morphologically abnormal embryos, and increased embryo loss compared to non-stressed control ewes (Dutt, 1963). Embryo loss for the combined 0- and 1- day groups was higher than in the 3- and 5- day ewes, suggesting that the sheep zygote is most sensitive to the harmful effects of high temperature during the initial stages of cleavage while in the oviduct. Eighty-four percent of the control ewes lambed compared with 10 % for ewes in the 0-and 1- day groups, 35 % in the 3-day group and 40 % in the 5-day group. In ewes not heat stressed until 5 days post breeding, embryo losses, and percentage of ewes which lambed, were similar to those of control ewes.

In vitro studies have further defined when the early embryo is most sensitive to thermal stress. Fertilized ova grown in vitro through the first cell division at 40 C and subsequently transferred to recipient ewes had a lower rate of survival than those grown at 38 C. As the period of culture at 40 C was delayed until either the second, third, or fourth cell division, differences in post-implantation death losses disappeared (Dutt, 1963).

Numerous studies have demonstrated the effects of heat stress, both in vivo and in vivo, on early embryonic development. Maternal heat stress during the initial stages of pregnancy results in extensive developmental retardation and/or arrest among stressed embryos. For example, development was retarded in embryos recovered about 50 hours after mating from mice that had been heat stressed (32 to 34 C) during the time period when embryos undergo first cleavage division (Elliott and Ulberg, 1971). Significantly more of these embryos were only in the two-cell stage compared to controls (21 C). When these embryos were cultured for an additional 48 hours, significantly fewer embryos of the heat-stressed group progressed in development to the blastocyst stage. High oviductal and uterine temperatures may have directly altered metabolic activity of stressed embryos, resulting in a reduced rate of embryonic growth and developmental retardation. In vitro, high temperature depresses synthesis of DNA and RNA (Elliott and Ulberg, 1971; Bellve, 1972; Sheean et al., 1974) and cellular proteins (Heikkila and Schultz, 1984) by blastocysts. Heat-stress induced decreases in RNA and protein synthesis may result in embryonic retardation and possibly delay or block cellular mitotic activity of stressed embryos.

The cause of heat-stress induced embryonic retardation and subsequent mortality is not known. Chromosomal abnormalities (Waldbieser and Chrisman, 1986) and congenital defects (Trujano and Wrathall, 1985; Mirkes, 1987) have been associated with heat stress. Oocyte meiotic maturation is extremely sensitive to hyperthermia and has been implicated as one of the causes of early embryonic death. Heat stress during the latter stages of oocyte maturation, just prior to ovulation strongly inhibits oocyte protein synthesis in vitro (Cerci et al., 1987) and has been shown to disrupt resumption of oocyte meiotic maturation to metaphase II and causes polar body retention in vivo in mice (Baumgartner and Chrisman, 1981a,b). Similarly, in vitro heat stress of bovine oocytes inhibited meiotic maturation (Lenz et al., 1983). An embryo resulting from fertilization of a heat-stressed and developmentally immature ovum, blocked at diakinesis-metaphase 1 or with retained polar body chromosomes, would most likely be genetically abnormal and would not likely survive. In fact, Baumgartner and Chrisman (1987) reported that exposure of mice to elevated temperature from the time of injection of an ovulating dose of hCG through the expected time of ovulation resulted in extensive pre- and post-implantation embryonic mortality. Staigmiller and Moor, (1984) reported that immature oocytes became fertilized and cleaved, but failed to progress in development to organized blastocyst stage embryos. Heat-induced inhibition of oocyte protein synthesis may result in the absence of proteins synthesized during oocyte maturation that have been reported to persist throughout early embryonic development and exert important regulatory roles in governing blastocyst formation (Moor and Trounson, 1977). Collectively, these results indicate that even though heat stressed oocytes are capable of being fertilized, residual effects of heat stress result in extensive developmental retardation and embryonic mortality.

Considerable research has focused on the effects of heat stress on the biochemical processes within the developing embryo. In response to heat shock, embryos generally display a major change in protein synthesis which is characterized by a decrease in overall protein synthesis and a concomitant increase in the sythesis of heat shock proteins (hsps). Of these proteins, the 70 kDa hsp is the most prominent hsp produced in vitro by mouse (Wittig et al., 1983) rat (Mirkes, 1987) and rabbit (Heikkila and Schultz, 1984a,b) morula/blastocyst stage embryos maintained at high incubation temperatures. While of uncertain function, hsps may play an essential role in cellular homeostasis and thermotolerance during periods of environmental stress (Loomis and Wheeler, 1980; Li and Werb, 1982).

Embryos are most sensitive to high environmental temperature during the initial cleavage stages (Dutt, 1983; Alliston et al., 1985) and become progressively thermotolerant with increasing age and cell number. This acquired thermotolerance is closely correlated with the ability of embryos to produce hsps in response to thermal stress (Muller et al., 1985; Heikkila et al., 1985) and appears to coincide with the species specific critical phase in embryonic development when the embryonic genome is activated and de novo transcription begins. In bovine embryos, this activation occurs at the 8- to 18-cell stage of development (Eyestone and First, 1988; Barnes et al., 1987).

Fetal-Placental Development During Late Pregnancy

Several reports indicate that thermal stress during late pregnancy in sheep causes marked reductions in uterine blood flow, decreased placental weights and retardation of fetal growth (Alexander and Williams, 1971, Brown and Harrison

1981; Brown et al., 1981; Alexander et al., 1987; Bell et al., 1987). Fetal stunting after heat stress in late pregnancy in sheep is associated with a marked reduction in the size of fetal cotyledons and maternal caruncles (Alexander and Williams, 1971; Alexander et al., 1987). This decrease in placentome size most likely results in decreased nutrient availability to the fetus. Contributing to this dilemma of the fetus is a decrease in umbilical and uterine blood flow (Bell et al., 1987) which further reduces nutrient availability to the developing fetus and probably contributes to fetal stunting. These effects are accompanied by reduced concentrations of fructose in fetal blood, as well as reduced placental glucose transfer capacity resulting in fetal hypoglycemia. Additionally, decreases in the PO_2 and oxygen saturation of fetal arterial blood and enlargement of the PO_2 difference between uterine and umbilical venous blood were observed (Bell et al., 1987). Collectively, these data clearly indicate that heat stress during late gestation alters placental function in relation to glucose and oxygen transport resulting in fetal growth retardation, fetal hypoxemia and hypoglycemia.

Approximately 60 % of the bovine fetal growth occurs during the last 90 days of pregnancy (Eley et al., 1978). Summer heat stress during this time period of fetal development in cattle results in reduced dry weights of total placental tissue (Head et al., 1981) and lower calf birth weights (Collier et al., 1980). These data clearly indicate that the late pregnant cow and fetus are sensitive to seasonal changes in environment.

In a designed experiment, Collier et al. (1982) examined the effect of a stressful thermal environment on conceptus and maternal functions during the periparturient period in dairy cattle exposed to a shade or no shade management system during the last trimester of pregnancy. No difference in gestation length was detected between shade and no-shade groups. Calf birth weight was lower in the no-shade group and cows in the no-shade group produced less milk at calving. The decrease in calf birth weight of no-shade cows was associated with a lower mean prepartum concentration of E_1SO_4 in maternal plasma. Since E_1SO_4 is produced by the cotyledon and was lower, it is an indication of reduced conceptus function during thermal stress. In addition, prepartum heat stress increased concentrations of PGFM postpartum and increased the rate of uterine involution (Lewis et al., 1984).

Summary and Conclusion

Successful preimplantation embryonic development and maintenance of pregnancy represents the culmination of numerous and complex endocrinological, morphological, physiological and immunological interactions between the developing conceptus and its mother. Survival of the embryo and associated maintenance of the corpus luteum are major biological limitations that contribute to the high rate of pregnancy failure that occurs in cattle prior to implantation. In tropical and subtropical areas of the world, normal levels of pregnancy failure are exacerbated during seasonal periods of high ambient temperature and relative humidity. Although various reproductive processes of the female may be affected adversely by an unfavorable thermal environment, early embryonic mortality may be the major factor causing depression of pregnancy rate in cattle. Research presented in this dissertation increases our understanding of the physiological and biochemical processes within the preimplantation bovine embryo and maternal unit that are affected by environmental heat stress. Such factors likely contribute to increased embryonic mortality in dairy cattle exposed to high ambient temperatures. From such knowledge, rational systems of reproductive management can be developed to reduce summer periods of infertility.

CHAPTER 2

EMBRYONIC DEVELOPMENT IN DAIRY CATTLE EXPOSED TO ELEVATED AMBIENT TEMPERATURE BETWEEN THE ONSET OF ESTRUS AND INSEMINATION

Introduction

Reproductive efficiency is depressed when cattle are maintained under environmental conditions of high ambient temperature and relative humidity. Pregnancy rates to artificial insemination are as low as 10 to 15 percent during July and August in the subtropical environment of Florida (Badinga et al., 1985).

The period of embryonic development most sensitive to heat stress appears to be between maturation of the oocyte and the initial cleavage stages of embryos (Alliston and Ulberg, 1961; Dutt, 1963; Alliston et al., 1965). For example, Dutt (1963) reported that elevated ambient temperature 24 h prior to fertilization had no effect on ova fertilization, but increased the incidence of embryonic abnormalities and altered conception rates of ewes. None of the ewes exposed to heat stress maintained pregnancy, whereas 85 % of control ewes lambed. Furthermore, Baumgartner and Chrisman (1987) observed extensive pre- and post-implantation embryonic mortality in mice heat stressed from the time of injection of an ovulating dose of hCG through the expected time of ovulation. Collectively, these results indicate that even though stressed oocytes are capable of being fertilized, residual effects of heat stress result in extensive developmental retardation and embryonic mortality. The cause of heat-stress induced embryonic mortality is not known Chromosomal abnormalities (Waldhbiser and Chrisman, 1986) and congenital defects (Trujano and Wrathall, 1985; Mirkas, 1987) have been associated with heat stress. Additionally abnormal maturation of oocytes prior to fertilization has been implicated as one of the causes of embryonic death (Baumgartner and Chrisman, 1987).

Since the early bovine embryo is sensitive to maternal heat stress (Dunlap and Vincent, 1971; Monty and Racowsky, 1987), it is important to determine the effects of maternal hyperthermia on bovine oocyte maturation and the role these affects may play in altering early embryonic development. The present experiment was designed to determine the effect of heat stress during oocyte maturation on subsequent embryonic development in superovulated dairy heifers.

Materials and Methods

Superovulation of Heifers

Sixteen, nulliparous Holstein heifers between 1.5 and 2 yrs of age were assigned randomly to either thermoneutral or heat stress treatments. Prior to superovulation, heifers were treated with a luteolytic injection of Lutalyse

(PGF$_2\alpha$; 25 mg, im, Upjohn, Kalamazoo, MI) between Days 6 and 15 of the estrous cycle. Beginning on Days 10 or 11 of the subsequent estrous cycle, heifers were superovulated by twice-daily injections of Follicle Stimulating Hormone (FSH-P, 32 mg total, im; Schering Co., Kenilworth, NJ) for 4 days, and were given Lutalyse (50 mg total, im) at the time of the 5th and 6th FSH-P injections (Table 2-1). Treatment with FSH-P was continued until heifers displayed behavioral estrus at approximately 48 h following Lutalyse. Heifer superovulatory response, i.e., number of ovarian follicles, was determined by real-time linear scanning ultrasound (LS-300 Ultrasound Diagnostic System, Tokyo Keiki Co. Ltd., Toyko, Japan) on Day 4 of FSH-P.

Environmental Treatments

Heifers which responded to superovulation (> 3 follicles, [≥ 10 mm], as determined by ultrasound on the last day of FSH treatment; n = 14) and observed in behavioral estrus were assigned randomly to either thermoneutral or hyperthermic environmental treatments. Control heifers (n=9) were exposed to a thermoneutral environment of 24 ± 2 C in an evaporatively-cooled barn. Heat-stressed heifers (n=7) were maintained for 10 h in a hyperthermic chamber environment where ambient temperature was 42 C and relative humidity (RH) was 75 %. This environment was sufficient to elevate rectal temperatures of heifers by 2.0 to 2.4 C, a hyperthermia similar to that found under natural conditions

Table 2-1 Superovulation, environmental treatment, artificial insemination and embryo recovery protocol for thermoneutral and hyperthermic heifers.

Day of Estrous Cycle	Event	Treatment Sequence
6 to 15	Palpation for CL	25 mg $PGF_2{}^{a}$
10	Superovulation: 5 mg FSH	at 0800 and 2000 h
11	4 mg FSH	at 0800 and 2000 h
12	3 mg FSH 25 mg $PGF_2{}^{a}$	at 0800 and 2000 h at 0800 and 2000 h
13	2 mg FSH	at 0800 and 2000 h
14	2 mg FSH	at 0800 and 2000 h
0	Estrus Environmental change for 10 h	Thermoneutral (24 C) vs Hyperthermic (42 C)
1	Artificial Insemination	
7	Nonsurgical Embryo Recovery	

in lactating dairy cows during the summer months (Roman-Ponce et al., 1977). An environmental chamber large enough to accommodate two heifers was used to impose heat stress treatment. Ambient temperature control in the chamber ranged from 2 to 50 C and was limited by a 4 C dew point and relative humidity range of 40 to 90 %. The environmental chamber was monitored (Honeywell recorder, Honeywell Co., Minneapolis, MN) on a continuous basis for alterations in ambient dry-bulb (DB) and wet-bulb (WB) temperatures during the experimental treatment sequence.

Respiration rates and rectal temperatures of all heifers were monitored at 2 h intervals during environmental treatments. Immediately following environmental treatments, both groups of heifers were maintained at thermoneutrality (24 ± 2 C) in the evaporatively-cooled barn and inseminated artificially at 15 and 20 h after the onset of estrus. Heifers were then managed under thermoneutral conditions for a 7 d period as provided by an environmental shade structure previously described by Roman-Ponce et al. (1977).

Embryo Recovery and Evaluation

Embryos were recovered nonsurgically on Day 7 after insemination following a procedure modified by Drost (1986). Embryos were flushed from the uterine lumen with Dulbecco's phosphate buffered saline (PBS; Gibco, Grand Island, NY) by intermittent gravity flow through a 2-way, round tip Foley catheter (16 to 20 French gauge; American Hospital Supply, Jacksonville, FL) introduced into the uterus. The flushing effluent was filtered through a 75 um EmCon embryo filter (EmCon filter, Immuno Systems, Biddeford, ME) which was then cleared into a flat bottom grid searching dish (Fisher Scientific, Orlando, FL). Embryos were located under a stereomicroscope (American Optical Corporation, Buffalo, NY) at 15 and 40 X magnification, and evaluated with a compound microscope (Nikon Company, Koyko, Japan) at 100 and 400 X magnification for morphological characteristics and stage of development utilizing several criteria as described previously (Elsden et al., 1978). Based on these criteria, embryos were given a stage classification score (Stage 1 - < 8 cells; 2 = 9-16 cells; 3 = early morula; 4 = compact morula; 5 = early blastocyst, 6 = blastocyst) and also a quality classification score (1 = excellent, perfect embryo; 2 = good, trivial imperfections such as a few small extruded cells; 3 = fair, definite but no severe problems such as moderate numbers of extruded cells or small amounts of degeneration; 4 = poor, partly degenerate or vesiculated cells, 5 = very poor; 6 = unfertilized ova).

Embryos examined by light microscopy were further grouped into three categories according to morphological criteria outlined by Greve and Lehn-Jensen (1979):

Normal- Embryos at the expected developmental stage (morulae to blastocysts) with normal morphology; morulae with a compact cell mass, blastocysts with a distinct blastocoele, trophoblast and embryonic disc.

Retarded/Abnormal- Embryos not at the expected stage of development (up to a maximum of 16 cells) with or without deviations in their morphological appearance (extruded or degenerate blastomeres or blastomeres of uneven size)

Unfertilized- Unfertilized, one-cell ova.

Embryos were evaluated by fluorescence microscopy to determine viability as described by Schilling et al. (1979) Embryos were incubated for 15 min at room temperature in 2 ml PBS containing 0.2 ml of fluorescent dye 4'-6'-diamidino-2-phenylindol (DAPI, 0.1 mg/ml in 0.9 % NaCl; Sigma Chemical Co., St. Louis, MO). Fluorescent DAPI stain has a highly sensitive affinity for DNA and is able to penetrate the nuclear membrane and stain nuclei of dead or degenerate blastomeres (Dann et al., 1971). Following staining, embryos were placed into PBS to eliminate background nonspecific fluorescence and examined under a light-reflected fluorescence microscope (Olympus Vanox, Olympus Optical Co. Ltd. Toyko, Japan) at 50 and 100 X magnification using an OG-530 and RG-12 barrier filter (Olympus Optical Co. Ltd.). The DAPI staining is characterized by a brilliant yellow-colored fluorescence of single nuclei (partial fluorescence) or of all nuclei (positive fluorescence) within an embryo.

Statistical Analyses

Effect of environmental treatments on respiration rate, rectal temperature and embryonic responses, i.e, stage of development, quality score and estimated number of cells per embryo, of heifers were analysed by standard least-squares analyses of variance utilizing the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Analyses were modeled with with cows nested in treatment. The statistical model was: Y_{ijk} = treatment$_i$ + treatment$_i$(cow$_j$) + error$_{ijk}$. Effect of treatment on categorical embryonic responses were analyzed by maximum-likelihood procedures using log-linear functions of independent variables and standard Chi-square procedures (SAS, 1985).

Results

Superovulation of Heifers

Superovulatory response and embryo recovery efficiency of heifers are presented in Table 2-2. There was no treatment affect on mean number of induced corpora lutea (9.7 ± 1.8), embryos recovered (4.0 ± .6) or the percentage of embryos recovered per heifer (45.4 %). Similarly, the percentage of heifers from which at least one ova or embryo was recovered (78.6 %) was not significantly influenced by heat stress. Mean number of follicles (9.0 ± 1.4) detected by ultrasound on Day 4 of FSH-P was highly correlated (P<0.01, r=.83) with mean number of corpora lutea determined per rectum at the time of embryo recovery.

Heat Stress

Environmental temperature change within the climatic chamber was sufficient to produce hyperthermia in heat-stressed heifers. Elevation in ambient temperature from 24 to 42 C induced an overall 203 % increase ($P<0.01$) in respiratory rate and a 2.4 C increase ($P<0.01$) in rectal temperature in hyperthermic compared to thermoneutral heifers throughout the duration of treatment (Table 2-3). Rectal temperatures of heifers increased gradually throughout heat stress, at a rate of 0.42 C/h during the first 6 h and a rate of 0.05 C/h during the final 4 h of heat stress (Figure 2-1). After the cessation of heat stress temperature treatments, when heifers were returned to an environmental temperature of 24 C, respiration rates and rectal temperatures decreased and were similar to thermoneutral heifers by 15 h and prior to artificial insemination.

Embryo Stage of Development

Embryos were recovered nonsurgically at Day 7 of pregnancy and evaluated microscopically. Stages of embryonic development and quality are presented in Tables 2-4 and 2-5. Overall, quality of embryos was reduced ($P<0.001$) and percentage of embryos classified as good to excellent was lower (4.0 vs 47.4 %, $P<0.01$) in heat-stressed heifers Embryos recovered from stressed heifers were retarded; thus, classified generally as being at an earlier developmental stage ($P<0.001$) when compared to the developmental stage of

Table 2-2. Superovulatory responses and embryo collection efficiency of thermoneutral and heat-stressed heifers.

Measure	N	Thermoneutral	Heat Stress
Heifers flushed	14	8	6
Heifers with embryos recovered[a]	11	8	5
Collection efficiency[b] (%)	78.6	75.0	83.3
Follicles	9.0 ± 1.4	9.8 ± 1.5	8.0 ± 1.8
Corpora lutea	9.7 ± 1.6	11.4 ± 2.2	8.0 ± 1.2
Embryos	4.0 ± .6	3.7 ± .7	5.0 ± .5
Embryo recovery[c] (%)	45.4	33.3	62.5

[a] Heifers from which no ova or embryos were recovered were excluded from analysis.

[b] Percentage of heifers flushed from which ova or embryos were recovered

[c] Percentage of embryos recovered relative to the number of corpora lutea palpated per rectum.

Table 2-3. Respiration rates and rectal temperatures of thermoneutral (control) and heat-stressed heifers (least squares mean ± se).

Treatment	N	Ambient temperature (C)	Respiration rate (per/min)	Rectal temperature (C)
Control	6	24	68 ± 7**	38.9 ± .2**
Heat stress	5	42	138 ± 4	41.3 ± .2
		24[a]	78 ± 2	38.8 ± .2

** P<0.01 for heat stress vs thermoneutral.

[a] Ambient temperature at the time of artificial insemination.

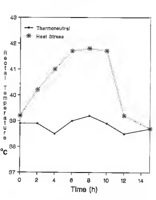

Figure 2-1. Mean heifer rectal temperature throughout the imposition of thermoneutral or heat stress environmental treatments.

embryos recovered from control heifers. Furthermore, estimated mean number of cells per embryo was lower (P<0.001) for embryos recovered from heat-stressed heifers.

Embryo Quality

The distribution of embryos classified as normal, retarded-abnormal or unfertilized ova differed (P<0.001) between heat stress and thermoneutral treatments (Table 2-6). A higher percentage of retarded and/or abnormal embryos were recovered from heat-stressed heifers (68.0 vs 21.1 %). The percentage of unfertilized ova recovered from heifers was similar between treatment groups.

Embryos classified into developmental and quality groups, when subjected to DAPI fluorescent staining, displayed different degrees of fluorescence reaction (Table 2-7). With normal embryos, 100 % were classified DAPI-negative (no cytoplasmic or nuclear staining). Embryos classified as retarded and/or abnormal showed a striking difference in staining as none of the embryos were DAPI-negative. A partial fluorescence was observed in 47.6 % of these embryos, whereas 52.4 % displayed a positive fluorescence reaction. Unfertilized ova showed a weak diffuse fluorescence (DAPI-positive) and were regarded as dead.

The distribution of embryos classified as fluorescent negative, partially positive and positive differed (P<0.005) between heat stress and thermoneutral treatments (Table 2-7).

Table 2-4. Developmental stage, quality, estimated cell number and degree of retardedness of embryos[a] recovered from thermoneutral and heat-stressed heifers (least squares mean ± se).

Evaluation	Thermoneutral	Heat stress**
N	19	25
Stage	3.1 ± .2	1.4 ± .2
Quality	2.9 ± .3	4.9 ± .3
Cells/Embryo	41.9 ± 4.8	10.9 ± 3.7
Retardedness (d)[b]	1.4 ± .3	3.4 ± .2

[a] Data for all embryos (normal, retarded-abnormal, and unfertilized) included in analysis.

** $P<0.001$, for heat stress vs thermoneutral.

[b] average number of days embryos were retarded at the time of recovery.

Table 2-5. Distribution* of embryos classified by light microscopy into developmental and quality groups as affected by environmental temperature.

Group	N	Treatment group Thermoneutral % (n)	 Heat stress % (n)
≤ 16 cells[a]	21	23.5 (4)	85.0 (17)
Morula	14	70.6 (12)	10.0 (2)
Blastocyst	2	5.8 (1)	5.0 (1)
Good to Excellent	10	47.4 (9)	4.0 (1)
Poor to Fair	27	42.1 (8)	76.0 (19)
Unfertilized ova	7	10.5 (2)	20.0 (5)

[a] Embryo group does not include unfertilized ova.

* $X^2 = 11.42$; $P<0.01$ distribution among developmental groups different between treatments.

* $X^2 = 11.56$; $P<0.01$ distribution among quality groups different between treatments.

Table 2-6. Distribution* of normal and retarded-abnormal embryos and unfertilized ova recovered from thermoneutral and heat-stressed heifers.

Treatment		Embryo		
		Normal	Retarded abnormal	Unfertilized ova
Thermoneutral	n	13	4	2
	%	68.4	21.1	10.5
Heat stress	n	3	17	5
	%	12.0	68.0	20.0

* X^2=15.05, P<0.001 distribution of embryos different between treatments.

Table 2-7. Distribution of 4'-6'-diamidino-2-phenylindol (DAPI)[a] fluorescence reaction of embryos classified by light microscopy into developmental and quality groups and as effected by environmental temperature.

Group	N	DAPI Reaction %** Negative % (n)	 Partial % (n)	 Positive % (n)
Normal	16	100.0 (16)	----------	----------
Retarded-Abnormal	21	----------	47.6 (10)	52.4 (11)
Unfertilized ova	7	----------	----------	100.0 (7)
Good to Excellent	10	100.0 (10)	----------	----------
Poor to Fair	27	22.2 (6)	37.0 (10)	40.7 (11)
Thermoneutral	19	68.4 (13)	21.1 (4)	10.5 (2)
Heat stress	25	12.0 (3)	24.0 (6)	53.5 (16)

[a] The DAPI staining is characterized by a brilliant yellow-colored fluorescence of single nuclei (partial fluorescence) or of all nuclei (positive fluorescence) within an embryo.

** X^2 = 15.05; P<0.001 distribution of fluorescence reaction different among developmental groups.

** X^2 = 31.53; P<0.001 distribution of fluorescence reaction of embryos different between quality groups.

** X^2 = 17.04; P<0.01 distribution of fluorescence reaction of embryos different between treatments.

A higher incidence of embryos that displayed partially positive and positive DAPI reactions were observed in heat stress (77.5 %) compared to thermoneutral (31.6 %) heifers.

Discussion

Physiological studies have demonstrated clearly the efficacy of exogenous gonadotrophins in stimulating ovarian follicular development in cattle (Elsden et al., 1978; Donaldson 1984; Moor et al., 1984 Lerner et al., 1987). In the present study, virgin heifers were used in order to maintain uniformity among animals with respect to age and parity. These factors have been shown to greatly influence superovulation rate in response to FSH-P in dairy cattle (Lerner et al., 1987). Ovarian stimulation with decreasing dosages of FSH-P, beginning at Days 10 or 11 of the estrous cycle, effectively enhanced follicular development in heifers. The large number of follicles detected by ultrasound was highly correlated with the subsequent number of corpora lutea at the time of embryo recovery.

Superovulation response varied among heifers and was expected based on the results of Monniaux et al. (1983) and Moor et al. (1984). They demonstrated that variability in responsiveness to superovulation in cattle may be due in part to differences in intraovarian factors such as the follicular population present on the ovaries at the time of superovulation. Ultrasound may provide an effective alternative to rectal palpation in estimating response to superovulation and potential embryo yields. Detection of follicular development immediately following superovulation treatment may increase efficiency and utilization of donor and recipient cattle. Nonsuperovulated donors may be eliminated prior to insemination, and a better estimate can be made of the number of recipients required at the time of embryo transfer.

Heifers maintained under hyperthermic conditions were heat stressed as indicated by a two-fold increase in respiratory rates and a 2.4 C elevation in rectal temperatures. Overall fertility (% normal embryos) for thermoneutral and heat-stressed heifers was 68.4 and 12.0 %, respectively. A similar suppression in fertility of dairy heifers maintained in hyperthermic environmental chambers (Putney et al., 1986) and lactating dairy cows during the summer months (Gwazdauskas et al., 1973; Badinga et al., 1985) has been associated with similar increases in rectal temperature.

Heifers were heat stressed for a 10 h period beginning at the onset of behavioral estrus. This period coincides with the early stages of nuclear and cytoplasmic maturation of the preovulatory oocyte (Hyttel et al., 1986). Oocyte nuclear maturation is considered to be the developmental progression from the appearance of the germinal vesicle of the arrested dictyate stage, through meiosis I to metaphase II, and extrusion of the first polar body (Hyttel et al., 1986; Moor and Gandolfi, 1987). Additionally, oocyte maturation is accompanied by quantitative and qualitative alterations in the synthesis of RNA (Rodman and Bachvarova, 1976) and proteins (Wassarman et al., 1981) essential for the continuation of meiosis and germinal vesicle breakdown (Mangia and Canipari, 1977; McGaughy and VanBlerkom, 1977; VanBlerkom and McGaughy, 1978). Hence, due to the experimental design, oocytes were exposed to high temperature in vivo during the maturation and reprogramming of obligatory events critical for successful oocyte fertilization and subsequent embryonic development (Warnes et al., 1977; Thibault, 1977).

Since fertilization rate was similar for thermoneutral and heat-stressed heifers, oocytes are capable of being fertilized and undergoing initial cleavage, despite exposure to an acute heat stress in vivo. Baumgartner and Chrisman (1987) reported no effect of maternal heat stress during oocyte maturation on subsequent fertilization in mice, and Dutt, (1963) observed that heat stress of ewes prior to fertilization had no effect on fertilization rate. In both instances however, exposure of oocytes to elevated temperature resulted in extensive pre- and post-implantation embryonic mortality.

Embryos recovered from thermoneutral heifers were at the expected developmental stage (early to late morulae) for Day 7 of pregnancy. Variation in stage of embryonic development within heifers was similar to that reported previously for superovulated cattle (Newcomb, 1982) and may be due, partially, to the extended time period during which ovulation and fertilization may occur in superovulated cattle (Maxwell et al., 1978; Angel, 1979; Yadav et al., 1985).

Maternal heat stress during oocyte maturation resulted in extensive developmental retardation and/or arrest of development of embryos. Embryonic development did not progress beyond the 8- to 16-cell stage by Day 7 of pregnancy in 85 % of embryos from stressed heifers. Heat stress-induced retardation and possible termination of embryonic development at the 8- to 16-cell stage coincides with the in vitro developmental block (Newcomb, 1982; Eyestone and First, 1986). This may be a critical phase in embryonic development during which the bovine embryonic genome is activated allowing transition from maternal to embryonic control of development (King et al., 1985).

Exposure of oocytes to high temperature during the early stages of nuclear maturation may result in the failure of embryos to undergo genomic activation and continued development to the compacted morula and expanded blastocyst stages. In support of this hypothesis, Elliott et al. (1968) and Elliott and Ulberg, (1971) reported that mouse embryos subjected to high temperature in vivo prior to and during the first cleavage division, failed, in most instances, to develop past the 2-cell stage. This coincides with the in vitro developmental block which occurs in mice (Goddard and Pratt, 1983).

If embryonic development is retarded due to heat stress, asynchrony between the periimplantation embryo and gravid uterine horn may occur. A uterine environment at an inappropriate stage for a delayed embryo may compromise embryonic viability and lead to subsequent embryonic death. Bovine embryo transfer studies have demonstrated clearly the need for embryo and uterine synchrony for embryonic survival and successful maintenance of pregnancy (Rowson et al., 1972; Wright, 1981).

The mechanism(s) by which heat stress of oocytes acts to delay or terminate subsequent embryonic development is not known. Oocyte meiotic maturation is extremely sensitive to hyperthermia and has been implicated as one of the causes of early embryonic death. Heat stress during the latter stages of oocyte maturation, just prior to ovulation strongly inhibits oocyte protein synthesis in vitro (Curci et al., 1987) and disrupts resumption of oocyte meiotic maturation to metaphase II and causes polar body retention in vivo in mice (Baumgartner and Chrisman, 1981a,b). Similarly, in vitro heat stress of bovine oocytes inhibited meiotic maturation (Lenz et al., 1983). An embryo resulting from fertilization of a heat-stressed and developmentally immature ova, blocked at diakinesis-metaphase I or with retained polar body chromosomes, would most likely be genetically abnormal and would not likely survive. Staigmiller and Moor, (1984) reported that immature oocytes became fertilized and cleaved, but failed to progress in development to organized blastocyst stage embryos. Heat-induced inhibition of oocyte protein synthesis may result in the absence of proteins synthesized during oocyte maturation that have been reported to persist throughout early embryonic development and exert important regulatory roles in governing blastocyst formation (Moor and Trounson, 1977).

Quality of embryos from stressed heifers was lower (fair to poor) and the embryos had a greater frequency of morphological imperfections. Imperfections such as the presence of blastomeres extruded from the cellular mass or degenerating blastomeres of irregular shape and with a dark granular appearance were often observed. Increased incidence of embryonic abnormalities due to maternal heat stress would, in all probability, result in increased rates of pregnancy failure as previously reported for sheep and cattle with elevated rectal temperatures at or near the time of mating (Ulberg and Burfening, 1967; Dunlap and Vincent, 1971; Gwazdauskas et al., 1973).

Fluorescence viability testing in combination with light microscopy enabled evaluation of the developmental capacity of embryos of different morphological classifications. Embryos classified by morphological criteria as developmentally normal were, in all instances, DAPI-negative and would most likely have continued to develop normally if transferred to recipients. The developmental capacity of retarded-abnormal embryos appears to be severely limited. Due to the intensity of DAPI staining, a proportion of the cells within each embryo were considered dead. Most of these embryos would likely fail to develop normally or survive. A higher incidence of positive DAPI reactions was observed in embryos from heat stressed heifers compared to control heifers. Increased fluorescence reactivity of heat stressed embryos reflects an increased incidence of degenerate or dead blastomeres observed by light microscopy.

In summary, superovulated Holstein heifers were exposed to environmental conditions of thermoneutrality (24 C) or hyperthermia (42 C) for 10 h from the time of onset of estrus (preovulatory period). On Day 7 of pregnancy, embryos were recovered nonsurgically and evaluated as to their morphological quality and developmental progress. Thermal stress had no effect on oocyte fertilization, but significantly increased the incidence of retarded embryos (fewer than 16 cells) with abnormal and degenerate cells. These data indicate that the preovulatory bovine oocyte is highly sensitive to thermal stress. Maternal heat stress during oocyte meiotic maturation prior to fertilization may severely compromise subsequent embryonic viability and lead to an increased rate of early embryonic mortality in cattle. This, in part, may be responsible for decreased reproductive efficiency in cattle during transient periods of high environmental temperatures. It is important to determine if bovine embryos continue to be sensitive to harmful effects if high ambient temperature during the initial stages of embryo development and blastocyst formation.

CHAPTER 3

EMBRYONIC DEVELOPMENT IN DAIRY CATTLE EXPOSED TO ELEVATED AMBIENT TEMPERATURES BETWEEN DAYS 1 TO 7 POST INSEMINATION

Introduction

Cattle maintained under environmental conditions of high ambient temperature and relative humidity are unable to dissipate environmental heat and often are hyperthermic with elevated body core temperatures. Increased temperature of the embryo's microenvironment, i.e. the oviduct and uterus, may adversely affect embryonic viability. For example, elevated rectal and uterine temperatures during the first few days after insemination are correlated closely with depressions in pregnancy rates in cattle (Ulberg and Burfening, 1967, Dunlap and Vincent, 1971; Gwazdauskas et al., 1973).

Specific periods during which the early bovine embryo is sensitive to heat stress have not been documented extensively. Dunlap and Vincent (1971) reported that elevated ambient temperature for 72 h immediately following breeding altered conception rates of beef heifers. None of the heifers exposed to heat stress maintained pregnancy, whereas 48 % of control heifers were pregnant.

It is important to determine if bovine embryos are sensitive to the harmful effects of high ambient temperature during the initial stages of embryonic development and blastocyst formation. This experiment was designed to determine the effect of thermal stress on early embryonic development in superovulated dairy heifers exposed to elevated ambient temperature and humidity between Days 1 to 7 after insemination.

Materials and Methods

Superovulation of Heifers

Twenty-nine, nulliparous Holstein heifers between 1 and 2 yrs of age were assigned randomly to either thermoneutral or heat stress treatments. Prior to placement in environmental chambers, a pre-experimental group of eight heifers were treated with a luteolytic injection of Lutalyse ($PGF_2\alpha$; 25 mg, im; Upjohn, Kalamazoo, MI.) between Days 6 and 15 of the estrous cycle. Four heifers on Day 7 of the subsequent-induced estrous cycle, were placed into two environmental chambers (see Chapter 2), each large enough to accommodate two heifers. Animals were maintained under daily thermoneutral conditions of 20 C and 70 % relative humidity (RH) for 3 days (Figure 3-1). Environmental chambers were monitored (Honeywell recorder, Honeywell Co., Minneapolis, MN.) on a continuous basis for alterations in ambient dry-bulb (DB) and wet-bulb (WB) temperatures during the experimental treatment sequence. A temperature humidity index (THI=

DB + .36WB + 41.5) was calculated and used as a comfort index as described by Buffington et al. (1981).

Following a 3-day period of chamber adaptation, heifers were superovulated by injections of follicle stimulating hormone (FSH-P, 32 mg total, im; Schering Co., Kenilworth, NJ.) and were given Lutalyse (50 mg total, im) on Day 3 of FSH-P (Table 3-1). Treatment with FSH-P was continued until heifers showed behavioral estrus at approximately 48 h following Lutalyse. Heifers were inseminated artificially three times at 10 h intervals beginning at 4 h after the onset of estrus.

Environmental Treatments

On the anticipated day of ovulation, 30 h after the onset of estrus, heifers were assigned to either thermoneutral or hyperthermic environmental treatments. Control heifers (n=15) were exposed to a thermoneutral chamber environment of 20 C and 70 % RH. Heat-stressed heifers (n=14) were exposed to a hyperthermic chamber environment maintained at 30 C and 70 % RH for 16 h and 42 C, 70 % RH for 8 h. This temperature change was sufficient to increase rectal temperatures by 2.0 to 2.5 C and simulate hyperthermia similar to that found under natural conditions in lactating dairy cows during the summer months (Roman-Ponce et al., 1977). Environmental temperatures for each treatment were applied for a 7-day period (Figure 3-1). Respiratory rates and rectal temperatures of all experimental

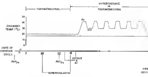

Figure 3-1. Schematic representation of superovulation, environment and embryo recovery treatment sequence imposed on heifers at various days of the estrous cycle.

Table 3-1. Superovulation, artificial insemination, environmental treatment and embryo collection protocol for experimental heifers.

Day of Estrous Cycle	Event	Treatment Sequence
6 to 15	Palpation	25 mg PGFα
7	Environmental change	Enter chambers at thermoneutrality (20 C)
10	Superovulation:	5 mg FSH at 0800 and 2000 h
11		4 mg FSH at 0800 and 2000 h
12		3 mg FSH at 0800 and 2000 h 25 mg PGFα at 0800 and 2000 h
13		2 mg FSH at 0800 and 2000 h
14		2 mg FSH at 0800 and 2000 h
0	Insemination at 4, 14 and 24 h after estrus	
1	Environmental change	Thermoneutral (20 C) vs. Hyperthermic (30 C and 42 C)
7	Nonsurgical Embryo Recovery	

heifers were monitored daily at 4 h intervals during imposition of environmental treatments. Experimental treatment sequence was replicated several times with subgroups of different heifers in order to obtain a total of 10 heifers per treatment group from which ova and/or embryos were recovered.

Embryo Recovery and Evaluation

Embryos were recovered nonsurgically on Day 7 of pregnancy following a modified procedure of Drost (1986) as described in Chapter 2. Embryos were located under a stereo-microscope (American Optical Corporation, Buffalo, NY.) at 15 and 40 X magnification and evaluated with a compound microscope (Nikon Company, Koyko, Japan.) at 100 and 400 X magnification. Embryos were evaluated based on morphological quality and stage of development utilizing several criteria as described previously (Elsden et al., 1978; see Chapter 2). Embryos were examined by light microscopy and grouped into four categories according to morphological criteria outlined by Greve and Lehn-Jensen (1979):

Normal- Embryos at the expected developmental stage with normal morphology; morulae with a compact cell mass, blastocysts with a distinct blastocoele, trophoblast and embryonic disc.

Abnormal- Embryos at the expected developmental stage but with deviations in their morphological appearance (extruded or degenerate blastomeres or blastomeres of uneven size).

Retarded- Embryos not at the expected stage of development (up to a maximum of 16 cells) with or without morphological abnormalities.

Unfertilized- Unfertilized, one-cell ova.

A proportion of the embryos (n=51, 34 %) categorized according to morphological criteria were further evaluated by fluorescence microscopy to determine viability as described in Chapter 2.

Statistical Analyses

Effect of environmental treatments on heifer respiration rate, rectal temperature and embryonic responses, i.e. stage of development, quality score and estimated number of cells per embryo, were analyzed by standard least-squares analysis of variance utilizing the General Linear Models procedure of the Statistical Analysis System (1985). Analyses were modeled with a nested design with cows nested in treatment. The statistical model was: Y_{ijk} = treatment$_i$ + treatment$_i$-(cow$_j$) + replication$_k$ + treatment$_i$*replication$_k$ + residual. Effect of treatment on categorical embryonic responses was modeled with maximum-likelihood procedures using log-linear functions of independent variables and standard Chi-square procedures (1985).

Results

Superovulation of Heifers

Superovulatory response and embryo recovery efficiency of heifers are presented in Table 3-2. There was no treatment effect on mean number of induced corpora lutea (11.2 ± 1.9), embryos recovered (7.5 ± 1.8) or the percentage of embryos recovered per heifer (66.7 %). Similarly, the percentage of heifers from which at least one ova or embryo were recovered (63.0 %) was not significantly influenced by heat stress.

Treatment Induced Hyperthermia

Environmental temperature change increased the temperature humidity index (THI) within climatic chambers and was sufficient to produce hyperthermia in heat-stressed heifers. Elevation in chamber ambient temperature from 20 (THI=66) to 30 C (THI=80) reduced a 64.3 % increase in respiratory rate ($P<0.01$), while a temperature of 42 C (THI=95) further increased both respiratory rate (277 %, $P<0.01$) and rectal temperature (2.4 C, $P<0.01$) in hyperthermic compared to thermoneutral heifers (Table 3-3).

Embryo Stage of Development

Stage of embryonic development and quality are presented in Table 3-4. Overall, embryonic quality was reduced ($P<0.001$) and percentage of embryos classified as good to excellent was lower (23.2 vs 57.3 %; $P<0.001$, Figure 3-2 and Table 3-4) in heat-stressed heifers. Embryos from stressed

Table 3-2. Least squares mean ± se superovulatory responses and embryo recovery[a] efficiency of thermoneutral and heat-stressed heifers.

Measure	N	Thermoneutral	Heat stress
Heifers flushed	29	15	14
Heifers with embryos recovered	20	10	10
Collection efficiency*(%)	69.0	66.7	71.4
Embryos	7.5 ± 1.8	6.8 ± 1.4	8.2 ± 1.7
Corpora lutea	11.2 ± 1.9	9.7 ± 1.3	12.6 ± 1.8
Embryo recovery (%)	66.7	70.1	65.1

[a] Heifers from which no ova or embryos were recovered were excluded from analysis.

* Percentage of heifers flushed from which ova or embryos were recovered.

Table 3-3. Least squares mean ± se for respiration rates and rectal temperatures of thermoneutral (control) and heat-stressed heifers.

Treatment	N	Chamber Temp. (C)	THI[a]	Respiration rate (per/min)	Rectal temperature (C)
Control	10	20	66 ± .2	40.7 ± .6	38.7 ± .1
Heat stress	10	30	80 ± .2**	66.9 ± 1.1**	38.7 ± .1
		42	95 ± .2**	153.5 ± .9**	41.1 ± .1**

[a] Temperature humidity index (THI).

** P<0.01 for heat stress versus thermoneutral.

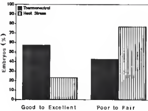

Figure 3-2. Distribution of embryo quality (grouped as good to excellent and poor to fair) between thermoneutral and heat-stressed heifers.

heifers were classified generally as being at an earlier developmental stage (P<0.05), thus retarded when compared to embryos recovered from control heifers. Estimated mean number of cells per embryo was also lower (P<0.001) for embryos recovered from heat-stressed heifers.

Embryo Quality

The distribution of embryos classified as normal, abnormal, and retarded, or as unfertilized ova differed (P<0.001) between heat stress and thermoneutral treatments (Table 3-5). A higher percentage of abnormal and retarded embryos was recovered from heat-stressed heifers (60.9 vs 29.4 %). The percentage of unfertilized ova recovered from heifers was similar between treatment groups. Among the 17 heifers providing embryos, the percentage classified as retarded plus abnormal was correlated (P<0.001, r=.63) with mean maximum daily rectal temperatures during daily exposure to 42 C (Figure 3-3).

Embryos classified as normal, abnormal and retarded, or as unfertilized ova, when subjected to DAPI fluorescent staining displayed different degrees of fluorescence reaction (Table 3-6). With normal embryos, 66.4 % were classified DAPI-negative (no cytoplasmic or nuclear staining), whereas 31.6 % displayed a partial fluorescence reaction. Embryos classified as abnormal and retarded showed a striking difference in staining as only 16.7 % of abnormal and none of the retarded embryos were DAPI-negative. However, partial

Table 3-4. Least squares mean ± se for developmental stage, quality, estimated cell number and degree of retardedness of embryos[a] recovered from thermoneutral and heat-stressed heifers.

Evaluation	Thermoneutral	Heat Stress
N	68	82
Stage	4.1 ± 0.3	2.4 ± 0.2**
Quality	2.5 ± 0.3	4.2 ± 0.3**
Cells/Embryo	69.5 ± 6.7	33.3 ± 5.5**
Development (d)	5.1 ± 0.4	3.8 ± 0.4**
Retardedness (d)[b]	1.9 ± 0.4	3.2 ± 0.4*

[a] Data for all embryos (normal, abnormal and retarded, or unfertilized ova) included in analysis.

* P<0.05; ** P<0.001, for heat stress vs thermoneutral.

[b] average number of days embryos were retarded at the time of recovery.

Table 3-5. Distribution* of normal, abnormal or retarded embryos and unfertilized ova collected from thermoneutral and heat-stressed heifers.

Treatment	Embryo category			
	Normal	Abnormal	Retarded	Unfertilized
Thermoneutral:				
n=68	35	9	11	13
%	51.47	13.24	16.18	19.12
Heat stress:				
n=82	17	22	28	15
%	20.73	26.83	34.15	18.29

* X^2=18.09; P<0.001 distribution of embryos different between treatments.

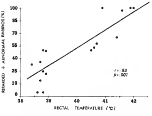

Figure 3-3. Linear effect of heifer rectal temperature on the percentage of retarded and abnormal embryos recovered at Day 7 of pregnancy. Data points represent individual heifer overall mean rectal temperature during periods of exposure to 20 or 42 C. Heifers (n=3) from which only unfertilized ova were recovered were not included in the analysis.

Table 3-6. Distribution of 4'-6'-diamidino-2-phenylindol (DAPI)[a] fluorescence reaction of embryos classified by light microscopy into four developmental groups as influenced by environmental temperature.

Group	N	DAPI Reaction %, (N)		
		Negative	Partial	Positive
Normal*	19	68.4 (13)	31.6 (6)	---------
Abnormal	12	16.7 (2)	83.3 (10)	---------
Retarded	8	---------	100.0 (8)	---------
Unfertilized	12	---------	----------	100.0 (12)
Thermoneutral**	24	45.8 (11)	33.3 (8)	20.8 (5)
Heat Stress	27	14.8 (4)	59.3 (16)	25.9 (7)

[a] DAPI stains dead nuclei of the embryo with a yellow fluorescence.

* $X^2 = 70.110$; $P<0.001$ distribution of fluorescence reaction different among embryo groups.

** $X^2 = 6.11$; $P<0.005$ distribution of fluorescence reaction different between treatments.

fluorescence was observed in 83.3 and 100 % of these respective embryos. Unfertilized ova showed a weak diffuse fluorescence (DAPI-positive) and were regarded as dead.

The distribution of embryos classified as fluorescent negative, partially positive and positive differed ($P<0.005$) between heat stress and thermoneutral treatments (Table 3-6). A higher incidence of embryos that displayed partially positive and positive DAPI reactions were observed in heat stress (85.2 %) compared to thermoneutral (54.2 %) heifers.

Discussion

Ovarian stimulation with decreasing dosages of FSH-P, effectively enhanced follicular development in heifers, as indicated by the large number of corpora lutea present at rectal palpation preceding embryo recovery. Although response varied among heifers, there was no significant treatment effect on heifer superovulatory response. The superovulation protocol was implemented during the pre-experimental adaptation period when environmental chambers for both treatment groups were maintained at thermoneutrality (20 C). Variability in heifer superovulatory response was expected based on the results of Monniaux et al. (1983) and Putney et al., (Chapter 2).

Embryo collection efficiency, expressed as the percentage of embryos plus ova recovered relative to the number of palpable corpora lutea, was not influenced by heat stress. Thus, presence of intact embryos or their ability to be sucessfully recovered from the uterine lumen does not appear to be altered by a potentially hyperthermic uterine microenvironment induced by heat stress.

Heifers maintained under hyperthermic conditions were heat stressed effectively as indicated by a four-fold increase in respiratory rates and a 2.4 C elevation in rectal temperatures. Overall fertility (% normal embryos) of control and heat-stressed heifers was 51.5 and 20.7 %, respectively. Such increases in rectal temperature have been associated with a similar suppression in fertility of lactating dairy cows during the summer months (Gwazdauskas et al., 1973; Badinga et al., 1985;). Elevated respiratory rate without a concomitant increase in rectal temperature occurred when stressed heifers were exposed on a daily basis to 38 C for 16 h. Consequently, heifers were able to dissipate completely the imposed thermal load by evaporative cooling processes (Curtis, 1983). This physiological response is similar to that observed in cattle exposed to milder heat stress under natural environmental conditions in the field (Curtis, 1983). A greater heat load at 42 C was necessary to push non-lactating heifers into a daily period of hyperthermia similar to that observed in lactating cows (Roman-Ponce et al., 1977).

Fertilization rates were similar in both treatment groups. Due to the experimental design, imposition of heat stress was not initiated until 24 to 36 h after observed estrus. This should have permitted sufficient time for fertilization to occur prior to heat stress. Increases in rectal and uterine temperatures prior to or at the time of fertilization are adversely associated with conception rate and embryonic quality in cattle (Gwazdauskas et al., 1973; Chapter 2). This may be due, in part, to direct harmful effects of elevated temperature on maturing and/or fertilized ova, resulting in death of the embryo before implantation (Ulberg and Burfening, 1967).

Embryos recovered from control heifers and subjected to morphological evaluation were generally considered to be at the expected developmental stage (early to late morula) for Day 7 of pregnancy. Rate of embryonic development appeared to be slowed considerably in heat stressed heifers. Stressed embryos were classified generally as being at an earlier developmental stage, hence retarded when compared to embryos recovered from control heifers. It is important to note that the average number of days embryos were estimated to be retarded (3.2 days) is similar to the total number of days heifers were exposed to severe heat stress (2.0 days) plus the inherent retardation (1.9 days) observed in thermoneutral heifers. Similar effects of heat stress on embryonic development have been observed in mice (Elliott and

Ulberg, 1971; Bellve, 1972). Maternal heat stress during the first 2 days of pregnancy resulted in extensive developmental retardation and/or arrest among embryos recovered from stressed females. High oviductal and uterine temperatures may have directly altered the metabolic activity of stressed embryos, resulting in developmental retardation and a reduced rate of embryonic growth. High temperature has been reported to depress synthesis of RNA (Elliott and Ulberg, 1971; Bellve, 1972) and cellular proteins (Heikkila and Gilbert, 1984; Putney et al., 1987) by blastocysts cultured in vitro. Decreased RNA and protein synthesis may result in embryonic retardation by possibly delaying or blocking cellular mitotic activity of stressed embryos.

A reduced rate of embryonic growth, may result in asynchronous development between the preimplantation embryo and gravid uterine horn. Normal embryonic development prior to implantation depends upon an appropriate sequence of changes in nutrient rich uterine secretions (Bazer, 1975). A maternal microenvironment at an inappropriate stage for a delayed embryo may compromise embryo viability and most likely lead to subsequent embryonic death. Bovine embryo transfer studies have demonstrated clearly the critical need for embryo and uterine synchrony for embryo survival and successful maintenance of pregnancy (Wright, 1981). For example, pregnancy rates were reduced 52.2 % when embryos collected on Day 7 after estrus were transferred to recipient dairy cattle on Days 4 to 5 of the estrous cycle (Rowson, 1972). Since the average degree of retardedness of all heat-stressed embryos was approximately 3 days, decreased pregnancy rates, similar to those obtained with asynchronous transfers, would be expected if stressed embryos were allowed to continue development in utero or transferred to recipients without regard to stage of embryonic development.

A higher incidence of abnormal embryos from heat-stressed heifers was observed at collection. Morphological imperfections such as the presence of blastomeres extruded from the cellular mass, or degenerating blastomeres of irregular shape and with a dark granular appearance were often observed. Increases in the incidence of embryonic developmental abnormalities was correlated positively with increases in rectal temperatures. Heifers which attained rectal temperatures greater than 41.5 C during extreme hyperthermia in all cases had embryos that were completely retarded and/or degenerate. Direct harmful effects of high temperature, as well as toxic effects of the oviductal and uterine microenvironment due to heat stress, probably account for much of the abnormalities observed at collection. Increased incidence of embryonic abnormalities would in all probability result in increased rates of pregnancy failure as previously reported for sheep and cattle with elevated rectal temperatures at or near the time of mating (Dunlap and Vincent, 1971; Gwazdauskas et al., 1973).

CHAPTER 4

HEAT STRESS INDUCED ALTERATIONS IN THE SYNTHESIS AND SECRETION OF PROTEINS BY DAY 7 BOVINE BLASTOCYSTS

Introduction

The preimplantation bovine embryo is extremely sensitive to harmful effects of high environmental temperature. Maternal hyperthermia retards embryonic development (Putney et al., 1986, Chapters 2 and 3) and results in decreased quality (Gordon et al., 1987) and viability (Monty and Racowsky, 1987) among stressed embryos. Bovine embryos are most sensitive to heat shock during the initial cleavage stages and appear to become progressively thermotolerant during the preimplantation period of blastocyst elongation and pregnancy recognition at Days 16 to 18 of pregnancy (Biggers et al., 1987).

Considerable research has focused on the effects of heat shock on the biochemical processes within the developing embryo. In vitro heat shock of morula/blastocyst stage mouse (Wittig et al., 1983) and rabbit (Heikkila and Schultz, 1984a) embryos reduces total protein synthetic capacity while enhancing the synthesis of heat shock proteins (hsp). Heat shock proteins may play an essential role in cellular homeostasis and thermotolerance during periods of environmental stress (Loomis and Wheeler, 1980; Li and Werb, 1982).

Since bovine embryos are most sensitive to maternal hyperthermia during the initial cleavage stages and appear to become progressively thermotolerant with increasing age, thermotolerance may be associated with stage-dependent hsp synthesis by the developing bovine embryo. This study examined whether heat shock, in vitro, alters protein synthesis and secretion by bovine blastocysts, to determine if heat shock-induced alterations in protein synthesis by embryos plays a role in early embryonic mortality.

Materials and Methods

Superovulation of Donors

Beef and dairy cattle (n=30) were treated with a luteolytic injection of Lutalyse (PGF$_{2}\alpha$; 25 mg; Upjohn, Kalamazoo, MI) between Days 6 and 15 of the estrous cycle. Beginning on Day 10 of the induced estrous cycle, heifers were superovulated as described in Chapter 3. Heifers were inseminated artificially following onset of estrus.

Embryo Collection

Embryos were recovered nonsurgically on Day 7 post estrus following a modified procedure of Drost (1986) as described in Chapter 2. Embryos were isolated using a stereomicroscope and subsequently transferred to PBS containing 20 % (v/v) heat-inactivated fetal calf serum (FCS, Fisher Scientific). Embryos were examined with a compound microscope at 100 X magnification and evaluated based on morphological quality and stage of development as described in Chapter 2. Good quality morula to blastocyst stage embryos (n=90) were subsequently cultured as described below

In Vitro Culture

Preparation of modified MEM was as described by Godkin et al. (1982) except that medium was additionally supplemented with 1 % (v/v) MEM vitamin mix (Gibco, Grand Island, NY). Embryos were cultured individually in 5 ml of MEM supplemented with 50 uCi L[^{35}S]methionine (specific activity ~500 mCi/mmol; Amersham Corporation, Arlington Heights, IL) and 20 % (v/v) FCS. Cultures were maintained under an atmosphere of 47.5 % O_2, 50 % N_2 and 2.5 % CO_2 (v/v/v) in the dark on rocking platforms. Thermoneutral cultures (n=45 embryos) were maintained at 38 C for 24 h. Heat-stressed cultures (n=45 embryos) were acclimated at 39 C for 6 h and then placed at 42 C for 18 h. Medium from 20 cultures (10 thermoneutral, 10 heat stress) was sampled (1 ml) at 6 and 24 h after initiation of culture. Samples of medium were stored at -70 C until assayed for incorporation of radiolabel into secreted proteins.

Preparation of Culture Medium and Embryos for Analyses

At termination of culture, embryos were isolated from culture medium, serially washed in PBS to remove unincorporated radiolabeled precursors, and examined for stage of development with a compound microscope at 100 X magnification. Embryos were stored individually in 10 ul PBS in 1.5 ml bullet tubes (Sarstedt, Princeton NJ) at -70 C.

Protein Determination

Incorporation of [^{35}S]methionine into secreted and intracellular proteins was determined by trichloroacetic acid (TCA) precipitation. Samples (50 ul) of conditioned media collected after 6 and 24 h of culture, or individual embryos (n=20, 10 thermoneutral, 10 heat stress) were placed onto Whatman 3MM paper (Whatman, Clifton, NJ), previously saturated with 20 % TCA (w/v) (Fisher Scientific) and allowed to dry. Precipitation of proteins onto filter paper and removal of nonproteinaceous compounds was accomplished by serial washings of the filter paper with 20 % TCA, 5 % TCA and 95 % ethanol as described by Mans and Novelli (1961). Radioactivity of precipitated protein was determined by scintillation spectrometry.

Electrophoresis

Aliquots of embryonic tissue, containing equal numbers of embryos (n=5) were solubilized in 5 % β-mercaptoethanol and 1 % sodium dodecyl sulfate by heating at 100 C for 3 min. A total of 40 embryos (20 thermoneutral, 20 heat stress) were subjected to one-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (1D SDS-PAGE) was performed using the buffer system of Laemmli (1970). Separation of proteins was by electrophoresis in

12.5 % (w/v) polyacrylamide gels. Gels were stained with Coomassie brilliant blue R-250, impregnated with sodium salicylate and fluorography was carried out using Kodak XAR-5 film.

Statistical Analyses

Effect of temperature treatment on embryonic protein synthetic capacity was analyzed by least-squares analysis of variance utilizing the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Data analyses were modeled with a nested design with embryos nested within treatment. The statistical model was Y_{ij} = treatment_i + $\text{embryo}_j(\text{treatment}_i)$ + residual.

Results

Quantitative Protein Synthesis and Secretion

The synthesis and secretion of [^{35}S]methionine labeled proteins released into culture medium and present in tissue samples is presented in (Table 4-1). Prior to initiation of heat shock, secretion of radiolabeled proteins by embryos within heat shock treatment groups were similar to that of thermoneutral embryos (824 ± 122 vs 680 ± 122 dpm/6 h). Elevation of incubation temperature from 39 to 42 C reduced embryo protein synthetic capacity, resulting in a 35.9 % ($P<0.05$) and 70.5 % ($P<0.001$) decrease in incorporation of [^{35}S]methionine into secretory and intracellular proteins, respectively. Microscopic evaluation of embryos, at the end

Table 4-1. Incorporations of [^{35}S]methionine into TCA precipitable protein in tissue and medium collected after 6 and 24 h of culture by Day 7 embryos cultured at thermoneutral (39 C) or heat shock (42 C) temperatures (Least squares mean ± sem).

Group	N	Radiolabeled protein (DPM)[a]		
		secreted/6h[b]	secreted/24h	tissue/24h
Thermoneutral	10	824 ± 122	13,107 ± 1,361*	7,780 ± 340**
Heat stress	10	680 ± 122	8,406 ± 1,361	2,296 ± 340

[a] Radiolabeled protein expressed as DPM/ per embryo.

[*] P<0.05, 35.9 % decrease in secreted radiolabeled proteins for thermoneutral vs heat-stressed embryos

[**] P<0.001, 70.5 % decrease in radiolabeled tissue proteins for thermoneutral vs heat-stressed embryos.

[b] 6 h culture at 39 C for embryos in both treatment groups

of culture, revealed that fewer (P<0.05) heat-stressed embryos progressed in development (42.8 %, 19 of 45 embryos) compared to control embryos (78.3 %, 35 of 45 embryos).

Qualitative Analysis of Proteins

Qualitative differences in [^{35}S]methionine incorporation into tissue proteins synthesized by embryos were evaluated. Electrophoretic examination (1D SDS-PAGE) of radiolabeled proteins revealed a complex spectrum of newly-synthesized, radiolabeled proteins. Representative fluorographs of protein patterns presented in Figure 4-1 are typical of all embryos analyzed. The predominant radiolabeled polypeptide detected appeared as a single band having an apparent molecular weight of 45 kDa. Several smaller and larger molecular weight species were detected by fluorography. Heat shock altered the array of proteins present in embryonic tissues. In particular, heat shock enhanced the synthesis of a 70 kDa protein (Figure 4-1).

Discussion

Physiological studies have demonstrated that the preimplantation embryo is extremely sensitive to high environmental temperatures (Alliston and Ulberg, 1961; Ulberg and Sheean, 1973; Putney et al., 1988; Chapters 2 and 3) The present study has shown that in vitro elevation of incubation temperature of Day 7 bovine embryos from 39 to

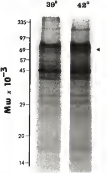

Figure 4-1. One dimensional polyacrylamide gel electrophoresis of proteins present in embryos cultured at 39 C or 42 C. Proteins were separated by SDS PAGE using 12.5% (w/v) polyacrylamide gels. Radiolabeled proteins were localized by fluorography. Equal numbers of embryos (n=5) were loaded per lane. Note the predominant heat-induced protein present in heat-shocked embryos are reduced in thermoneutral embryos (arrow, = 70 kDa).

42 C reduces total protein synthetic capacity while enhancing the synthesis of a heat shock protein (hsp). Similar effects of thermal stress on in vitro protein production by Day 17 embryos have been noted in cattle (Putney et al., 1987; Chapter 7) and swine (Wetteman et al., 1984). In addition to altering embryonic protein synthesis, preliminary data with limited numbers of Day 7 embryos (n=5) suggests that heat shock alters DNA synthesis of stressed embryos. Embryos (n=3) cultured for 24 h at 42 C incorporated 71.2 % less [^{3}H]thymidine into DNA than embryos (n=2) cultured at 39 C (58.8 vs 204.3 DPM/24 h/embryo). However, due to limited embryo numbers, no significant effect of treatment on incorporation of [^{3}H]thymidine was detected. These in vitro data suggest that high environmental temperature may alter embryo metabolic activity in vivo, and lead to reduced growth rates and embryonic retardation.

Embryos synthesized numerous intracellular radiolabeled polypeptides. A predominant protein species of 45 kDa was detected in tissues of embryos. This protein species most likely represents -actin synthesized during early embryonic development (Moor and Gandolfi, 1987). Other smaller and larger molecular weight protein species were detected by fluorography, but they were not the major intracellular proteins. Embryos maintained at heat shock incubation temperatures displayed enhanced synthesis of a 70 kDa hsp, similar to that produced in vitro by heat-shocked mouse (Wittig et al., 1983) rat (Mirkes, 1987) and rabbit (Heikkila and Schultz, 1984a,b) embryos. In response to heat shock embryos display a major change in protein synthesis which is characterized by a decrease in overall protein synthesis and a concomitant increase in the synthesis of hsps. Of these proteins, the 70 kDa hsp is the most prominent. While of uncertain function, hsps may play an essential role in cellular homeostasis and thermotolerance during periods of environmental stress (Loomis and Wheeler, 1980; Li and Werb, 1982).

Mammalian embryos are most sensitive to high environmental temperature during the initial cleavage stages (Dutt, 1963, Alliston et al., 1965) and become progressively thermotolerant with increasing age and cell number. Thermotolerance is closely correlated with the ability of embryos to produce hsps (Muller et al., 1985; Heikkila et al., 1985) and coincides with the critical phase in embryonic development when the embryonic genome is activated and de novo gene transcription begins. In bovine embryos, this activation occurs at the 8- to 16-cell stage of development (Eyestone and First, 1986; Barnes et al., 1987). Therefore, by the morula to blastocyst stage of development bovine embryos are able to synthesize hsps in response to heat shock and, as a result, may be less sensitive to acute high environmental temperatures compared to earlier cleavage stage embryos, i.e. those with less than 8 to 16 cells.

In summary, the effects of heat shock on protein synthesis of the Day 7 embryos were examined in vitro. Elevation in tissue incubation temperature from 39 to 42 C induced a dramatic reduction in embryo protein synthesis and secretion and enhanced the synthesis of a 70 KDa heat shock protein. These in vitro results suggest that exposure of pregnant cows to high environmental temperature and humidity, as often occurs during summer months of the year, may alter embryonic gene expression and synthesis of proteins involved in early embryonic development. Heat shock protein synthesis by morula/blastocyst stage embryos suggests that by day 7 of pregnancy embryos have acquired some degree of thermotolerance and may be able to survive acute increases in uterine temperature as often occurs when cattle are hyperthermic and unable to dissipate environmental heat load. Embryo transfer at Day 7 postestrus, at a time when embryos may be less sensitive to high temperatures, may increase pregnancy rates in heat-stressed cattle.

CHAPTER 5

INFLUENCE OF SUMMER HEAT STRESS ON REPRODUCTIVE PERFORMANCE OF BOVINE EMBRYO DONORS AND RECIPIENTS

Introduction

The early bovine embryo is extremely sensitive to harmful effects of maternal hyperthermia. Exposure of dairy cows to summer heat stress conditions (Gordon et al., 1987; Monty and Racowsky, 1987) or dairy heifers to hyperthermic (42 C) environmental chambers (Putney et al., 1986, Chapter 3), during the first 7 days of pregnancy, resulted in decreased quality (Gordon et al., 1987) and viability (Monty and Racowsky, 1987) of embryos recovered from cows and an increased incidence of abnormal or retarded embryos recovered from heifers (Putney et al., 1986, Chapter 3).

Bovine embryos continue to be sensitive to high temperatures beyond the period of early cleavage and blastocyst formation. Short term exposure of pregnant beef cattle to thermal stress (37 C, 40 % RH) during the period of rapid blastocyst development and pregnancy recognition (Days 8 to 16 after insemination) reduced conceptus weights and resulted in a trend for lower incidence of successful pregnancy (50 % vs 83 %) compared to control heifers maintained at 22 C, 25 % RH (Biggers et al., 1987).

These results indicate that heat stress during the period of blastocyst elongation and pregnancy recognition altered embryonic development, and this may lead to increases in early embryonic mortality. However, thermal sensitivity after Day 7 does not appear to be as great as during the earlier period of development before Day 7.

Environmental modifications such as various shade structures, air conditioning, and evaporative cooling have been used to improve fertility of cattle during summer heat stress (Thatcher et al., 1983). Reproductive management strategies should be combined with environmental modification to maximize conception rates. For example, embryo transfer on Day 7 of pregnancy may be a means of bypassing the early developmental period of embryonic thermal sensitivity. Consequently, it is important to determine the effects, if any, of environment on reproductive performance of embryo donor and recipient cattle. This study is a retrospective analysis of detailed superovulation and embryo transfer records, collected over a 4 year period during the operation of a commercial embryo transfer program at Medina Valley Veterinary Services, Castroville, Texas, to evaluate the effect of environmental temperature on embryo donor and recipient cattle.

Materials and Methods

Donor and Recipient Cattle

During a 4 year period from 1981 to 1984, nonsurgical embryo recoveries were performed on 3,908 superovulated donor cows of various beef (99.4 %, n=3885) and dairy (0.6 %, n=23) breeds. Embryo transfers were performed on 19,936 recipient cows (93.8 %, n=18705) and heifers (5.4 %, n=1068) of various beef (92.6 %, n=18,453) and dairy (7.4 %, n=1320) breeds. Females of beef breeds were predominantly Angus, Hereford, Limousin, Simmental, Brahman, Brangus and Beefmaster. Females of dairy breeds were Holstein, Jersey and Brown Swiss. Donors were maintained and embryos were recovered primarily (72.3 %, n=2825) on client farms located at 47 different geographical locations throughout the southwestern region of the United States including Texas, Oklahoma and Mississippi, with the remainder of donor animals (27.7 %, n=1082) being managed at Medina Valley Veterinary Services located in Castroville, Texas. Recipient cattle were maintained and embryo transfers performed predominantly (74.9 %, n=14,933) on the client's farm.

Superovulation

Donor cows were superovulated by twice daily intramuscular injections of 5 mg follicle-stimulating hormone (FSH-P, Burns-Biotec, Ohmaha, NE) beginning on Days 8 to 12 of the extrous cycle (Day 0 = day of estrus). Cows received Lutalyse (PGF $_2\alpha$, 50 mg, im; Upjohn Company, Kalamazoo, MI) on

Days 2 or 3 of FSH-P treatment. Treatment with FSH-P was continued until donor cows displayed behavioral estrus at approximately 48 h following Lutalyse. Donor cows were inseminated artificially three times at 12 h intervals beginning 8 to 12 hours after the onset of estrus.

Embryo Recovery

Embryos were recovered nonsurgically on Days 6 to 8 post estrus by repeated flushings of the uterine lumen with Dulbecco's phosphate buffered saline (PBS) containing 2 % heat-inactivated fetal calf serum (FCS) through a two-way Foley catheter (C.R. Bard, Inc. Murray Hill, NY) introduced into the uterus. Embryos were identified and examined at 15 to 40 X magnification under a stereomicroscope and subsequently transferred to PBS containing 20 % FCS, 100 IU penicillin, 100 mcg streptomycin and 0.25 mcg amphotericin B per ml PBS. Embryos of transferable quality were aspirated individually into a 0.25 cc inseminating straw. The straws were heat sealed and placed horizontally in a test tube surrounded by a 500 ml water jacket, maintained at ambient temperature or within a range of 5 to 20 C, for transport to the transfer location. Donor animals were flushed and embryos recovered primarily (93.1 %, n=3638) by the same individual.

Embryo Evaluation

Embryos were evaluated based on stage of development and morphological characteristics. Transferrable embryos were classified as to stage of development utilizing the following definitions:

Morula- An embryo containing at least 16 cells that have not yet coalesced together to form a compact cellular mass.

Advanced Morula- An embryo containing more than 16 cells that have coalesced together to form a compact mass but without evidence of a blastocoele.

Early Blastocyst- An embryo with a blastocoele just starting to appear.

Blastocyst- An embryo with a large blastocoele and pronounced differentiation of the outer trophoblast layer and inner cell mass.

Advanced Blastocyst- A blastocyst that has expanded to fill the entire perivitelline space and has distinct thinning of the zona pellucida.

Hatched Blastocyst- A blastocyst undergoing the process of hatching or that has completely shed the zona pellucida.

Transferable embryos were further evaluated and graded based on morphological characteristics. Good embryos were considered normal for their respective stages of development with few if any detectable morphological defects. Fair embryos had small imperfections such as the presence of extruded or degenerate cells. Poor embryos had severe defects such as extruded degenerate cells and large vesicles but still contained a cellular mass that appeared viable.

Embryo Transfer

Recipient cattle had their estrous cycles synchronized with Lutalyse (25 mg, im) and observed twice daily for the onset of behavioral estrus. Cattle which displayed estrus within 5 days following Lutalyse were considered as having drug induced synchrony as opposed to natural synchrony where estrus was not associated with a PGF injection. Cattle were used as recipients indiscriminately regardless of whether they came into estrus naturally or following injection of Lutalyse. Prior to transfer of an embryo, all recipients were palpated rectally for the presence of a corpus luteum. Embryos were transferred nonsurgically into synchronized recipient cattle using a Cassou inseminating gun (M. Cassou, L'Aigle, France) which was inserted into the vagina and passed through the cervix up to the uterine horn ipsilateral to the ovary containing the active corpus luteum. Embryos were transferred primarily (97.9 %, n=19517) by the same individual. Recipient pregnancy diagnosis was determined by rectal palpation 40 to 60 days following transfer.

Climatological Data

Donor and recipient cattle were maintained at Medina Valley Veterinary Services or on client farms located at various geographical locations throughout the southwest region of the United States including Texas, Oklahoma and Mississippi. Environmental data collected during a 4 year period from 1981 to 1984 for regional locations where cattle were managed (Dallas, San Antonio, Waco, and Whichita Falls, Texas, and Meridian, Mississippi) were obtained from the National Oceanic and Atmospheric Administration (Ashville, NC). Mean maximum environmental ambient temperature during periods of early embryonic development of donors (Days 0 to 7 post insemination) and recipients (Days 0 to 10 post transfer) was determined to assess effect of environment on reproductive responses of cattle.

Statistical Analyses

Effect of environmental temperature on embryonic continuous responses, i.e. mean numbers of ova, fertilized embryos and embryos of transferable quality were analyzed by standard least-squares analysis of variance utilizing the General linear Models procedure of the Statistical Analysis System (1985). Effect of environment on categorical embryonic responses, i.e. stage of development and morphological quality, and recipient pregnancy rate were modeled with maximum-likelihood procedures using log-linear functions of independent variables and standard Chi-square procedures (1985). Interactions between environmental temperature and recipient breed type (dairy vs beef), parity (cow vs heifer), and lactational status (lactating vs dry) on pregnancy rate were analyzed by Chi-square procedures (1985).

Results

Donor Superovulation

Donor superovulatory responses and morphological characteristics of ova and embryos recovered nonsurgically on days 6 to 8 post estrus are presented in Tables 5-1 and 5-2. Donor collection efficiency, expressed as the percentage of animals flushed from which at least one ovum was recovered was 89.0 %. Cattle from which ova were recovered yielded a mean of 12.2 ± .3 ova per donor. A fertilization rate of 75.6 % resulted in a mean of 9.2 ± 2 embryos per donor. Of total ova recovered, 56.7 % were considered to be embryos of adequate morphological quality to be transferred into recipient animals, and of these transferable embryos, 85.5 % were subsequently transferred nonsurgically into recipients.

The effect of mean maximum environmental temperature, at the farm location where donor cattle were managed prior to embryo recovery, on donor reproductive performance and embryo stage of development and quality is presented in Tables 5-3 and 5-4. Mean daily maximum temperature which occurred during the first week of embryonic development (Days 0 to 7 post-estrus) had no effect on overall donor embryo collection efficiency, nor the mean number of ova collected, ova fertilized or embryos of transferable quality.

Embryo Stage of Development

Although a wide range of embryonic developmental stages were observed at collection within and among donors, embryos

Table 5-1. Donor superovulatory response and characteristics of embryos recovered nonsurgically.

Group	N	Mean/Donor[a]	(%)
Donor cattle	3,908		
Collection efficiency[b]	3,485		89.0
Ova collected	42,428	12.2 ± .3	100.0
Ova fertilized	32,076	9.2 ± .2	75.6
Embryos transferable	24,052	6.9 ± .2	56.7
Embryos transferred	20,635	5.9 ± .2	48.6

[a] Donor cattle from which no ova were recovered were excluded from data analysis.

[b] Donor collections (%) with at least one ovum recovered.

Table 5-2. Distributions[a] of embryo morphological quality by stage of development for embryos recovered from donor cattle.

Stage of development	N (% of total)	Embryo quality		
		Good	Fair	Poor
Embryo total	20,410	48.2	40.4	11.4
Morula	106 (0.5)	9.4	63.2	27.4
Advanced morula	9,676 (47.4)	35.3	43.7	21.0
Early blastocyst	5,967 (29.1)	42.9	52.7	4.4
Blastocyst	3,241 (15.8)	76.7	23.0	0.4
Advanced blastocyst	1,427 (6.9)	94.8	5.1	.05
Hatched blastocyst	46 (0.2)	86.2	10.3	3.5

[a] X2 = 145.23 P<0.001, distribution of embryo quality different among embryo developmental stages.

Table 5-3. Characteristics of embryos recovered from donor cattle maintained at various environmental temperatures[a]

Group		Environmental temperature (C)			
		<27	27-32	32-40	>40
Donor cattle		901	914	1,264	301
Collection efficiency[b]	(%)	(90.6)	(87.8)	(90.0)	(87.5)
Ova collected		12.7 ± .3	11.8 ± .3	12.1 ± .3	11.8 ± .6
Ova fertilized		9.5 ± .2	8.8 ± .2	9.3 ± .2	9.1 ± .5
	(%)	(74.7)	(74.6)	(76.6)	(77.2)
Embryos transferable		6.3 ± .2	6.5 ± .2	7.1 ± .2	6.8 ± .4
	(%)	(56.2)	(55.1)	(58.2)	(57.4)

[a] Mean daily maximum environmental temperature during the first week of embryonic development (Days 0 to 7 post estrus).

[b] Donor collections (%) with at least one ovum recovered.

were generally considered to be at the appropriate developmental stages for Days 6 to 8 of pregnancy. Embryos were recovered predominantely on Day 7 (90.8 %, n = 18,532) or prior to Day 7 (5.4 %, n = 1,102) post estrus. Hence, 77.0 % of embryos morphologically evaluated were considered to be at the morula to early blastocyst stages of development.

Embryo Quality

The distribution of transferable embryos classified as either good, fair or poor morphological quality differed (P<0.001) among the various embryonic stages of development (Table 5-2). A higher proportion of embryos at the earlier developmental stages (morula to early blastocyst) were graded as being poor or fair quality, displaying varying degrees of morphological defects such as extruded degenerate cells and large vesicles. Developmentally advanced embryos, i.e., blastocysts to hatched blastocysts, were predominantly of good quality and considered normal for their respective stages of development with few if any detectable morphological defects. The distribution of transferable embryos evaluated and grouped according to developmental stage and morphological quality was not influenced by ambient temperature.

Superovulation and Donor Breed Interactions

The effect of donor breed type, i.e., dairy vs beef, on superovulatory response and the morphological characteristics

Table 5-4. Percent distribution of embryo stage of development and quality score of embryos recovered from donors maintained at various environmental temperatures[a].

Group	N	Environmental temperature (C)			
		<27	27-32	32-40	>40
Embryo total	20,282	28.0	25.6	37.3	9.2
EMBRYO STAGE					
Morula	104	0.7	0.5	0.5	0.3
Advanced morula	9,593	47.4	47.5	47.2	47.3
Early blastocyst	5,901	27.1	29.9	29.9	30.7
Blastocyst	3,221	15.7	16.8	15.5	15.3
Advanced blastocyst	1,417	9.0	5.2	6.9	6.4
Hatched blastocyst	46	0.1	0.2	0.1	0.1
EMBRYO QUALITY					
Good	9,757	47.8	49.5	48.6	45.2
Fair	8,164	40.3	40.0	39.8	43.7
Poor	2,313	11.9	10.5	11.6	11.2

[a] Mean daily maximum environmental temperature during the first week of embryonic development (Days 0 to 7 post estrus).

of embryos is presented in Table 5-5. The mean number of ova collected was lower (P<0.05) for dairy than for beef donors (7.8 ± 2.0 vs 12.2 ± .2), resulting in a similar reduction in the mean number of fertilized ova and embryos of transferable quality recovered from dairy donors. Fertilization rate of ova as well as the percentage of embryos that were considered to be transferable were similar between breed types. Similarly, embryo quality was not influenced by the breed type of the donor from which embryos were recovered.

Interactions between donor breed type and environmental temperature on donor reproductive performance and embryo characteristics are presented in Tables 5-7 and 5-8. Reproductive performance of dairy cattle was severely compromised when donors were managed at farm locations were the mean daily maximum temperature exceeded 32 C during the time period between superovulation and embryo recovery. The mean number of ova recovered (1.3 ± 0.2), percentage of ova fertilized (20.0 %) and embryos of transferable quality (20.0 %) were lower (P<0.01) for dairy cattle managed under ambient temperatures greater than 32 C, compared to dairy cattle managed at locations when maximum temperatures did not exceed 32 C (Table 5-6).

The effect of beef donor breed type, i.e. Brahman vs European, on superovulatory response and the morphological characteristics of embryos is presented in Table 5-8. The mean number of ova collected was higher (P<0.05) for Brahman

Table 5-5. Characteristics of embryos recovered from dairy and beef donor cattle.

Group	Dairy N	Dairy Mean/Donor	Dairy (%)	Beef N	Beef Mean/Donor	Beef (%)
Donor cattle[a]	22			3,463		
Collection efficiency[b]			(88.0)			(83.2)
Ova collected	172	7.8 ± 2.0*		42,256	12.2 ± .2*	
Ova fertilized	134	6.1 ± 1.5*	(77.9)	31,942	9.2 ± .1*	(75.6)
Embryos transferable	96	4.4 ± 1.1*	(55.8)	23,956	6.9 ± .1*	(56.7)
EMBRYO QUALITY						
Good	47		(54.6)	9,710		(48.2)
Fair	30		(34.9)	8,134		(40.4)
Poor	9		(10.5)	2,304		(11.4)

[a] Donor cattle from which no ova were recovered were excluded from analysis.

[b] Donor collections (%) with at least one ovum recovered.

* P<0.05 significantly different between Dairy and Beef.

Table 5-6 Characteristics of embryos recovered from dairy and beef donor cattle maintained at various environmental temperatures[a]

Group	Environmental temperature (C)			
	<27	27-32	32-40	>40
DAIRY	8	10	4	0
Collection efficiency[b]	(88.9)	(83.3)	(100.0)	
Ova collected	9.0 ± 3.4	9.5 ± 3.3	1.3 ± .2**	
Ova fertilized (%)	7.8 ± 2.6 (87.5)	7.0 ± 2.4 (73.7)	0.3 ± .2** (20.0)	
Embryos transferable (%)	6.5 ± 2.2 (72.2)	4.3 ± 1.4 (45.3)	0.3 ± .2** (20.0)	
BEEF	893	904	1,260	301
Collection efficiency	(90.7)	(87.9)	(90.0)	(87.5)
Ova collected	12.7 ± .3	11.8 ± .3	12.2 ± .3	11.8 ± .6
Ova fertilized (%)	9.5 ± .3 (74.6)	8.8 ± .3 (74.6)	9.3 ± .2 (76.6)	9.1 ± .5 (77.2)
Embryos transferable (%)	7.2 ± .2 (56.1)	7.5 ± .2 (55.2)	7.1 ± .2 (58.2)	6.8 ± .4 (57.4)

[a] Mean daily maximum environmental temperature during the first week of embryonic development (Days 0 to 7 post estrus).

[b] Donor collections (%) with at least one ovum recovered.

** P<0.01 for dairy cattle in environmental group 32-40 C vs dairy cattle in groups < 27 and 27-32 C.

Table 5.7. Characteristics of embryos recovered from beef donor cattle of Brahman or European breed types maintained at various environmental temperatures[a]

Group	Environmental temperature (C)			
	<27	27-32	32-40	>40
Brahman	509	565	749	163
Collection efficiency[b]	(90.7)	(88.8)	(88.5)	(87.2)
Ova collected	13.1 ± .5	12.7 ± .5	12.6 ± .4	13.3 ± .5
Ova fertilized (%)	9.6 ± .4 (73.5)	9.1 ± .4 (72.0)	9.6 ± .3 (75.7)	9.9 ± .7 (74.6)
Embryos transferable (%)	7.2 ± .3 (54.7)	6.5 ± .3 (51.4)	7.1 ± .3 (56.5)	6.9 ± .5 (51.8)
European	347	268	351	131
Collection efficiency	(91.1)	(87.0)	(71.9)	(89.1)
Ova collected	12.2 ± .4	11.0 ± .5	11.8 ± .4	10.1 ± .7
Ova fertilized (%)	9.3 ± .4 (76.2)	9.1 ± .4 (82.7)	9.2 ± .3 (83.6)	8.3 ± .6 (82.2)
Embryos transferable (%)	7.3 ± .3 (59.8)	7.2 ± .4 (65.4)	7.2 ± .3 (65.4)	6.8 ± .6 (67.3)

[a] Mean daily maximum environmental temperature during the first week of embryonic development (Days 0 to 7 post estrus).

[b] Donor collections (%) with at least one ovum recovered.

Table 5-8, Characteristics of embryos recovered from beef donor cattle of Brahman[a] or European[b] breed types.

Group	Brahman			European		
	N	Mean/donor	(%)	N	Mean/donor	(%)
Donor Cattle[c]	2,035			1,238		
Collection efficiency[d]			(88.9)			(90.4)
Ova collected	26,043	12.8 ± .2[*]		14,298	11.5 ± .2	
Ova fertilized	19,282	9.5 ± .2	(74.0)	11,318	9.1 ± .2	(79.2)
Embryos transferable	14,112	6.9 ± .2	(54.2)	8,911	7.2 ± .2	(62.3)
Embryo Quality[**]						
Good	5,506		(45.5)	3,965		(52.2)
Fair	5,082		(42.0)	2,877		(37.9)
Poor	1,509		(12.5)	755		(9.9)

[a] Brahman, Brangus and Beefmaster Breeds.

[b] Angus, Hereford, Limousin and Simmental Breeds.

[c] Donor cattle from which no ova were recovered were excluded from analysis.

[d] Donor collections (%) with at least one ovum recovered.

[*] P<0.05 significantly different between Brahman and European donors.

[**] Chi Square 89.2; P<0.001, distribution of embryo quality different between Brahman and European donors.

than for European breed donors (12.8 ± .2 vs 11.5 ± .2). However, the mean number of ova fertilized and embryos of transferable quality were similar between breed types. The distribution of transferable embryos grouped according to morphological quality was influenced by breed type. A higher ($P<0.001$) incidence of good quality embryos were recovered from European breed donors.

In contrast to dairy donors, superovulatory response and embryo quality of beef donors (Brahman or European, Table 5-7) did not appear to be adversely affected by elevated environmental ambient temperature.

Recipient Pregnancy Rates

Recipient pregnancy rates, determined by rectal palpation 40 to 60 days following embryo transfer, were influenced by the developmental stage and quality of embryos nonsurgically transferred (Table 5-9). Pregnancy rates were lower (27.9 %, $P<0.01$) when embryos at the morula developmental stage were transferred to recipients as compared to pregnancy rates following the transfer of either advanced morula or any of the different blastocyst stage embryos. Pregnancy rates decreased ($P<0.01$) as the quality of embryos transferred decreased and were 33.7 % lower following transfer of poor compared to good quality embryos.

The effect of donor and recipient estrous cycle synchronization at the time of embryo transfer on subsequent recipient pregnancy rates is presented in Table 5-10.

Table 5-9. Recipient pregnancy rates following embryo transfer of embryos of various developmental stages and quality scores.

Group	N	(%)	Pregnancy rate (%)
Embryo total	19,936		55.7
EMBRYO STAGE[a]			
Morula	104	0.5	27.9
Advanced morula	9,449	47.4	50.2
Early blastocyst	5,808	29.1	59.1
Blastocyst	3,149	15.8	83.4
Advanced blastocyst	1,378	6.9	84.5
Hatched blastocyst	46	0.2	56.5
EMBRYO QUALITY[b]			
Good	9,598	48.1	64.6
Fair	8,054	40.4	52.4
Poor	2,284	11.5	30.9

[a] $X^2 = 52.30$ $P<0.01$, pregnancy rate different among embryo stage of development.

[b] $X^2 = 72.50$ $P<0.01$, pregnancy rate different among embryo quality score.

Pregnancy rate was highest (63.8 %) when embryos were transferred to recipients displaying estrus 12 to 24 h prior to that of donors. Recipient pregnancy rates decreased ($P<0.01$) as the degree of estrus synchrony decreased. Pregnancy rates decreased sooner and to a greater extend as the degree of estrous cycle sychrony decreased for embryos transferred to recipients in estrus after donors compared to recipients with the same degree of relative asynchrony but in estrus prior to donors.

The effect of environmental temperature at the farm locations where recipient cattle were managed after embryo transfers were performed on subsequent 40 to 60 day pregnancy rates is presented in Table 5-11. Overall mean monthly pregnancy rate (55.7 %) was not significantly influenced by the average ambient temperature which occurred during the month of the year when embryos were transferred.

In order to further assess affect of environment on recipient pregnancy rates, interactions between ambient temperature and recipient breed type (dairy vs beef), parity (cow vs heifer), or lactational status (lactating vs dry) were determined and are presented in Table 5-12. Overall mean pregnancy rate was higher ($P<0.001$) for dairy (60.5 %) than for beef (55.4 %) recipients. A higher pregnancy rate for dairy recipients was due primarily to a 23.1 % increase ($P<0.01$) in pregnancy rates of dairy heifers compared to beef heifers (63.1 vs 48.5 %, respectively). Overall pregnancy

Table 5-1[illegible] [illegible] recipient pregnancy rate [illegible] recipient with donor

Recipient-Donor Synchrony (h)		N	Pregnancy rate (%)
-72 to -96		29	27.6
-48 to -72	Recipient estrus	129	52.0
-24 to -48	before donor	610	60.3
-12 to -24		5,390	63.8
0 ± 12		9,849	55.2
+12 to +24		3,149	48.5
+24 to +48	Recipient estrus	734	35.5
+48 to +72	after donor	44	30.9
+72 to +96		19	22.3

[a] $\chi^2 = 357\ 06$, $P<0.001$, pregnancy rate different among synchrony groups.

Table 5-11. Pregnancy rate of recipient cattle as influenced by average monthly environmental temperature during the months of the year[a]

Month	N	Pregnancy rate (%)	Temperature (C)
Jan	1,753	59.8	7.8
Feb	1,577	53.2	9.7
Mar	1,627	52.4	14.0
Apr	1,892	54.1	18.5
May	2,010	55.1	22.6
Jun	2,302	55.8	26.7
Jul	2,435	58.2	28.6
Aug	1,530	59.2	25.1
Sep	970	52.9	19.6
Oct	951	55.6	19.6
Nov	900	57.7	13.3
Dec	1,996	54.4	9.1

[a] 1980-1984

[b] Average monthly temperature

Table 5-12. Pregnancy rate of recipient cattle maintained at various environmental temperatures[a].

Group	N	Pregnancy rate (%)[b]				
		Mean	Environmental temperature (°C)			
			<27	27-32	32-40	>40
Recipient cattle	19,936	55.7	54.9	55.6	55.3	61.6
Dairy	1,328	60.5	56.5	56.0	66.7	67.1
Cow	643	57.8	51.2	57.3	61.9	72.7
Lactating	199	53.7	58.9	60.9	63.1	66.7
Dry	444	59.7	58.6	54.8	61.4	75.9
Heifer	677	63.1	60.3	54.3	73.2	65.6
Beef	18,453	55.4	54.7	55.6	54.6	60.7
Cow	18,062	56.6	54.6	55.8	54.9	61.2
Lactating	4,618	57.0	57.9	55.3	54.8	65.8
Dry	13,444	55.1	54.0	55.6	54.5	57.8
Heifer	391	48.5	59.0	47.8	42.4	37.1

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[a] Mean daily maximum environmental temperature during embryonic development (Days 0 to 10 post transfer).

[b] See results for significant differences.

rate of Brahman breed type recipients was similar to that of European beef recipients (54.3 % vs 56.2 %, respectively). Interactions between environmental temperature, recipient breed type and parity were detected. Pregnancy rates increased (P<0.01) as the mean daily maximum ambient temperature, which occurred during days 0 to 10 post embryo transfer, increased. This increase was greater (P<0.01) for dairy than for beef cows as ambient temperature increased above 32 C (13.0 vs 1.8 %, respectively). In contrast, pregnancy rates decreased (P<0.01) for beef heifers and increased (P<0.01) for dairy heifer recipients as temperatures increased. A 51.3 % higher (P<0.01) pregnancy rate for dairy compared to beef heifers was detected when ambient temperatures were greater than 40 C (65.6 vs 37.1 %).

Discussion

Superovulation and embryo transfers were performed on donor and recipient cattle that were exposed to a wide range of environmental ambient temperatures. Average ambient temperatures, ranging from an average of 7.8 C during the month of January to 28.8 C during the month of July were observed. Since previous research has demonstrated clearly that the early bovine embryo (Days 0 to 17 of development) is sensitive to the harmful effects of maternal hyperthermia (Putney et al., 1986; Biggers et al., 1987), the present superovulation and embryo transfer records were analyzed for environmental effects that may be associated with this critical time period in early embryonic development. The mean daily maximum ambient temperatures which occurred during donor superovulation and embryonic development prior to embryo recovery (Days 0 to 7 post estrus) or temperatures which occurred during embryonic development after transfer to recipients (Days 0 to 10 post transfer) were determined and used as a measure of maternal environmental stress.

Overall donor superovulatory response, ova fertilization rate and the percentage of embryos of transferable quality were consistent with those in previously published reports which utilized donors of similar breed types and parity, and similar gonadotrophin treatments (Elsden et al., 1976; Schneider et al., 1980; Hasler et al., 1987). Superovulation response varied considerably among donors and was expected based on the results of Lerner et al. (1987). They demonstrated that variability in responsiveness to superovulation in cattle may be due, in part, to factors such as the age of the donor, the dose of follicle stimulating hormone administered, or the day of the estrous cycle when superovulation is started. Superovulated donors of dairy breeds produced an average of 4 to 5 fewer embryos per donor than animals of beef breeds, possibly indicating a lower responsiveness to ovarian stimulation with the gonadotrophin treatment utilized. Comparisons of superovulatory rates among different donor breeds indicate that differences in responsiveness to gonadotrophins exist relative to differences in the number of ova recovered among breed types (Shea et al., 1976; Brennan and Beehan, 1976).

Embryos recovered from donor cattle and subjected to morphological evaluation were generally considered to be at the expected developmental stage (morula to early blastocyst) for Days 6 to 8 of pregnancy. A high degree of variability was observed in embryo morphological development and quality within and among donors. Embryos recovered from superovulated cows often display a wide range of developmental stages (Newcomb and Rowson, 1980). Such variability may be due to an extended time period during which ovulation and fertilization may occur in superovulated cattle (Maxwell et al., 1978; Angel, 1978; Yadav et al., 1985).

Variation in environmental temperatures within the regions of farm locations where donor cattle were managed prior to embryo recovery, had no association with superovulatory response and embryo quality of beef cattle donors. A similar lack of environmental, or seasonal, effects on reproductive function of beef donors managed in a commercial embryo transfer unit have also been reported (Lerner et al., 1987; Critser et al., 1988; Massey and Oden, 1984; Shea et al., 1984). In contrast, superovulated dairy cows exposed to thermal stress produced fewer ova, and yielded a lower percentage fertilized ova and embryos of transferable quality. Furthermore, Monty and Racowsky (1987) reported decreased viability and developmental capacity of embryos recovered from superovulated heat-stressed Holstein cows. Decreased reproductive performance of dairy compared to beef donor cattle is probably due to an inability to maintain a normal body temperature under heat stress conditions because of a high rate of internal heat production associated with milk production.

The quality and developmental stage of embryos appears to be an important factor in determining the success of embryo transfer. Recipient pregnancy rates increased as the quality of embryos transferred increased, with excellent and good embryos yielding the highest pregnancy rates. Pregnancy rates were lowest when morulae were transferred to recipients and increased as the developmental stage of the transferred embryo progressed to the advanced blastocyst stage. Similar effects of embryo grade and stage of development on subsequent recipient pregnancy rates have been reported (Schneider et al., 1980; Hasler et al., 1987; Wright, 1981). Increased pregnancy rates of developmentally advanced embryos may be due to the fact that a higher proportion of these embryos were graded as good quality. Good quality embryos are often transferred first and to the most synchronous recipients, leaving poorer quality embryos to be transferred last and often to less closely synchronized recipients. As a result, the effect of embryo stage of development and quality score on recipient pregnancy rates may be amplified due to poorer quality embryos being held in culture longer and confounded by the degree of estrous cycle synchrony between recipients and donors.

An important factor in the success of embryo transfer is the degree of estrous cycle synchrony between the donor and recipient animal. Reports indicate that a ± 1.5 to 2.0 day asynchronous transfer can be tolerated without a significant reduction in pregnancy rate (Hasler et al., 1987; Seidel, 1981; Lindner and Wright, 1983). In the present study, pregnancy rates were higher when recipients were in estrus as much as 36 to 48 h before the donor animal and were lower when recipients were in estrus more than 12 h after the donor. This apparent advantage to the recipient in estrus prior to the donor has been observed previously (Hasler et al., 1987; Halley et al., 1979). The reasons for such an advantage are unclear. It has been suggested (Schneider et al., 1980; Hasler et al., 1987, Seidel, 1981) that embryos recovered from superovulated cattle may be slightly advanced in development relative to those from nonsuperovulated animals due to the possibility that ovulation and fertilization occur earlier, making the superovulated embryo older relative to the onset of estrus.

The effect of environmental ambient temperature at the farm location where recipients were managed after embryo transfers were performed was determined. Although pregnancy rates varied slightly, there was no significant effect of month or the average monthly ambient temperature on overall mean pregnancy rates of recipients. Elevated environmental temperature does not appear to adversely affect pregnancy rates of recipient cattle managed intensely in a commercial embryo transfer unit. In contrast, pregnancy rates of artificially inseminated cows range as low as 10 to 15 % during summer heat stress conditions (Gwazdauskas et al., 1973; Ingraham et al., 1974; Badinga et al., 1985).

In conclusion, failure to detect any adverse effects of summer heat stress on recipient pregnancy rates suggests that management of recipient cattle minimized any heat stress affects on fertility and/or that the early bovine embryo (Days 1 to 7 of pregnancy) is extremely sensitive to harmful effects of maternal hyperthermia. Embryo transfer at Day 7 post estrus, with good quality transferable embryos, may bypass this developmental period of embryonic sensitivity and provide an alternative to artificial insemination to circumvent heat stress-induced infertility in cattle. However, designed experiments are required to confirm this hypothesis.

CHAPTER 6

INFLUENCE OF SUMMER HEAT STRESS ON PREGNANCY RATES OF LACTATING DAIRY CATTLE FOLLOWING EMBRYO TRANSFER OR ARTIFICIAL INSEMINATION

Introduction

Embryo transfer may provide an alternative to artificial insemination to circumvent summer-time depression of pregnancy rate in cattle. Putney et al. (1987; Chapter 5) failed to detect any summer depression in pregnancy rates of recipient cattle managed in a commercial embryo transfer program. This retrospective study suggested that management of donor and/or recipient cattle minimized any heat stress effects on fertility, or transfer of good quality embryos to stressed recipients at Day 7 post estrus may bypass the period of embryonic development (Day 0 to 7 post insemination) when embryos are most sensitive to maternal hyperthermia. This designed experiment tested the potential advantage of embryo transfer on pregnancy rates of lactating dairy cows compared to cows inseminated during summer months in the subtropical environment of Florida

Materials and Methods

Superovulation

Holstein heifers (n=55) were managed on two commercial dairies, North Florida Holsteins and Richardsons Dairy, located in North Central Florida, USA. Heifers were treated with a luteolytic injection of Lutalyse (PGF$_2\alpha$; 25 mg, im, Upjohn, Kalamazoo, MI) between Days 6 and 15 of the estrous cycle. Beginning on Day 10 of the induced estrous cycle, heifers were superovulated as described in Chapter 2. Heifers were inseminated artificially at 4 and 14 hours following detection of estrus and subsequently managed under modified summer environmental conditions provided by shade structures. Heifers were superovulated in small groups (n=5) and each superovulation and embryo recovery treatment sequence repeated several times (North Florida Holsteins, n=6; Richardsons Dairy, n=5) during the period of May 29 to Sepetember 10, 1987.

Embryo Recovery and Evaluation

Embryos were recovered nonsurgically on Day 7 post estrus following a modified procedure of Drost (1986) as described in Chapter 2. Embryos were isolated under a stereomicroscope (American Optical Corporation, Buffalo, NY) at 15 and 40 X magnification and subsequently transferred to PBS containing 20 % heat-inactivated fetal calf serum (FCS).

Embryos were examined with a compound microscope (Nikon Company, Koyko, Japan) at 100 and 400 X magnification and evaluated based on stage of development (see Chapter 5) and morphological characteristics *Excellent* embryos were considered normal for their respective stages of development with no detectable morphological defects. *Good* embryos had trivial imperfections such as a few extruded cells. *Fair* embryos had imperfections such as small numbers of extruded or degenerate cells but still contained a cellular mass that appeared viable. *Poor* embryos had severe defects such as numerous extruded degenerate cells and large vesicles.

Embryo Transfer and Artificial Insemination

A total pool of recipient lactating Holstein cows (n=550) were synchronized for estrus with Lutalyse (25 mg, im) and observed twice daily for the onset of behavioral estrus. Recipient cows were synchronized for estrus in groups (n=50) and each synchronization treatment sequence repeated several times (North Florida Holsteins, n=6; Richardsons Dairy, n=5) during the period of May 29 to September 10, 1987. Prior to transfer of an embryo, all recipients were palpated per rectum for presence of a corpus luteum. Embryos of transferable quality were aspirated individually into a 0.25 cc insemination straw. Embryos were transferred nonsurgically into synchronized recipient cows (n=113) using a Cassou insemination gun (M. Cassou, L'Aigle, France) which was passed through the cervix and into the uterine horn ipsilateral to the ovary containing the active corpus luteum.

Potential recipient cattle not presented for embryo transfer and a second contemporary pool of lactating Holstein cows (n=524) were observed twice daily for the onset of behavioral estrus and inseminated with frozen semen within 12 h of detection of estrus on the same two farms.

After embryo transfer or artificial insemination, cows were managed under routine management conditions of the farms and exposed to summer heat stress environmental conditions. Milk samples were collected at 21 days post estrus, and progesterone (P4) concentrations determined for diagnosis of pregnancy via a competitive immunoassay procedure (Hoechst-Roussel Agri-Vet Company, Somerville NJ) which utilized a bovine P4 antibody linked to alkaline phosphatase. Standard curves were prepared with known amounts of bovine radioinert P4 (0 to 30 ng/ml). Concentrations of P4 in samples (20 ul) of whole milk were determined by colorimetric analysis via a spectrophotometer (Bio-Tek Instruments Inc. Winooski, VT) at a wave length absorbance of 492 nm. Inter- and intra-assay coefficients of variation for a reference sample (15 ng/ml) were 18.3 and 12.2 %, respectively. Concentrations of milk P4 greater than 10 ng/ml were considered to indicate the presence of a functional corpus luteum and cows were diagnosed as tentatively pregnant. A subsequent confirmation for pregnancy was made per rectum et approximately [illegible] days post estrus.

Climatological Data

Environmental data collected during a 5 month period [illegible] through September, 1987) for Gainesville Florida, located midway between the two farm locations where experimental animals were maintained, was obtained from the Department of Agronomy, University of Florida. Mean maximum environmental ambient temperature during periods of early embryonic development of donor heifers and artificially inseminated cows (Days 0 to 7 post insemination) and recipient cows (Days 0 to 10 post transfer) was determined to assess effect of environment on reproductive responses of cattle.

Statistical Analyses

Effect of mean maximum ambient temperature on continuous responses, i.e. mean numbers of ova, fertilized embryos and embryos of transferable quality were analyzed by standard least-squares analysis of variance utilizing the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Effect of environmental temperature on categorical embryonic responses, i.e. stage of development and morphological quality were modeled with maximum-likelihood procedures using log-linear functions of independent variables and standard Chi-square procedures (SAS, 1985). Effect of maximum environmental temperature on pregnancy rates of inseminated and recipient cows were modeled with maximum-likelihood procedures using log-linear functions of independent variables and standard Chi-square procedures (SAS, 1985).

Results

Superovulation of Heifers

Heifer superovulatory response and morphological characteristics of recovered ova and embryos are presented in Tables 6-1 and 6-2. The mean number of induced corpora lutea estimated by palpation per rectum at the time of embryo recovery was 9.6 ± 1.1 per donor. Ova and/or embryos were recovered from 78.2 % of heifers flushed, yielding a mean of 6.0 ± 0.8 ova and/or embryos per donor. A fertilization rate of 69.1 % resulted in a mean of 4.3 ± 0.7 recovered embryos per donor. Of total embryos recovered, 69.2 % were considered to be of adequate morphological quality to be transferred into recipient cows. Due to limited availability of recipients, 65.7 % of transferable embryos were subsequently transferred nonsurgically into recipient cows.

Embryo Stage of Development and Quality

Although a wide range of embryonic developmental stages were observed at collection within and among donors, embryos were considered generally to be at the appropriate developmental stages for Day 7 of pregnancy (Table 6-2). Developmentally, 55.1 % of embryos were considered to be at the morula to early blastocyst stages of development. The [illegible] Superovulatory responses and characteristics of embryos recovered nonsurgically from donor Holstein heifers.

Group	N	Mean/Donor	(%)
Donor heifers	55		
Collection efficiency[a]	43		78.2
Corpora lutea	529	9.6 ± 1.1	
Ova recovered	330	6.0 ± 0.8	
Ova fertilized	234	4.3 ± 0.7	69.1[b]
Embryos transferable	162	2.9 ± 0.6	49.1[c]
Embryos transferred	113	2.1 ± 0.6	34.2[d]

[a] Donor collections (%) with at least one ovum recovered

[b] Percent of total ova recovered that were fertilized

[c] Percent of total ova and/or embryos recovered that were considered embryos of transferable quality

[d] Percent of total ova and/or embryos recovered that were transferred

Table 6-2. Distribution[a] of embryo morphological quality by stage of development for embryos recovered from donor heifers.

Stage of development	N (% of total)	Embryo quality			
		Excellent	Good	Fair	Poor
Embryo total	234	36.7	20.1	11.1	32.1
< 16 cells	62 (26.5)	0.0	0.0	0.0	100.0
Morula	51 (21.8)	33.3	27.4	21.6	17.6
Advanced morula	52 (22.2)	28.8	36.5	25.0	9.6
Early blastocyst	26 (11.1)	61.5	34.6	3.8	0.0
Advanced blastocyst	43 (18.4)	88.4	9.3	2.3	0.0

[a] $\chi^2 = 291.48$ $P<0.001$, distribution of embryo quality different among embryo developmental stages.

quality distribution of embryos classified as either excellent, good, fair or poor differed ($P<0.001$) among stages of development. A higher proportion of embryos at the earlier developmental stages (morula to advanced morula) were graded as being of poor or fair quality, displaying varying degrees of morphological defects such as extruded degenerate cells and large vesicles. Embryos with ≤ 16 cells were considered severely retarded and in most instances contained degenerate cells. Developmentally mature blastocysts were predominantly of excellent quality and considered normal for their respective stages of development with few if any detectable morphological defects.

The effect of mean maximum ambient temperature during the 7-day period between insemination and embryo recovery on heifer reproductive performance and stage of embryo development and quality are presented in Table 6-3 and Figures 6-1 and 6-2. Mean weekly maximum ambient temperatures varied from 31.6 ± 1.0 to 34.9 ± 0.7 during May 29 to September 10, 1987. Mean maximum temperature had no effect on the mean number of induced corpora lutea, ova recovered, the percentage of ova fertilized, and embryos of transferable quality. In contrast, the distribution of embryos grouped according to developmental stage and morphological quality was influenced by temperature. Mean maximum temperatures were correlated ($r^2 = .66$, $P<0.01$) with increased percentage of embryos classified as ≤ 16 cells and

Table 6-3. Superovulatory responses and characteristics of embryos recovered from heifers as influenced by mean maximum environmental temperature during embryonic development (Days 0 to 7 post-insemination)

Group	N	Distribution by Environmental Temperature			
		< 32.2		≥ 32.2	
Corpora lutea	529	8.5 ± 1.6		10.4 ± 1.5	
Ova recovered	330	5.8 ± 1.3	(68.8)	6.1 ± 1.1	(58.9)
Ova fertilized	234	4.3 ± 1.2	(73.4)	4.1 ± 0.8	(66.3)
Embryos trans[a]	162	3.5 ± 1.1	(60.2)	2.3 ± 0.7	(42.1)
EMBRYO STAGE (%)*					
< 16 cells	62	19.2		31.8	
Morulas[b]	103	53.4		37.0	
Blastocysts[c]	69	27.3		31.1	
EMBRYO QUALITY (%)*					
Excellent to good	133	59.6		54.8	
Fair to poor	101	40.4		45.2	

[a] embryos of transferable quality

[b] morula and advanced morula combined

[c] early and advanced blastocyst combined

* $X^2 = 12.7$ P<0.01, distribution of embryo stages different between temperature categories

* $X^2 = 2.1$ P<0.10, distribution of embryo quality different between temperature categories

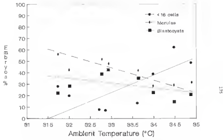


Figure 6-1. Linear effect of mean maximum ambient temperature which heifers were exposed to during the 7-day period between insemination and embryo recovery on the percentage of embryos classified by developmental stage as being (1) ≤ 16 cells, (2) morulae or (3) blastocysts.

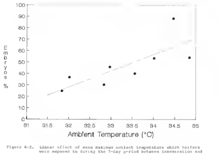


Figure 6-2. Linear effect of mean maximum ambient temperature which heifers were exposed to during the 7-day period between insemination and embryo recovery on the percentage of embryos classified by quality as being fair to poor

decreased percentage of those classified as morulae (Figure 6-1). Similarly, maximum temperature is correlated (r^2 = 51, P<0.05) with an increased percentage of embryos graded as poor to fair quality (Figure 6-2). A higher percentage of embryos graded as poor to fair quality and embryos with ≤ 16 cells were recovered from heifers during exposure to maximum ambient temperatures greater than 32 C for the 7-day period prior to embryo recovery (Table 6-3).

Pregnancy Rates of Lactating Cows

The effect of summer heat stress, measured as mean maximum ambient temperatures during the 7-day period following embryo transfer or artificial insemination on pregnancy rates of lactating cows is presented in Table 6-4. Overall mean pregnancy rates, determined by milk progesterone concentrations on Day 21 post estrus, were higher (P<0.001) for recipient than for inseminated cows (47.6 % vs 18.0 %, respectively). Similarly, pregnancy rates, determined by rectal palpation between 40 to 60 days following embryo transfer or insemination were higher (P<0.001) for recipient than for inseminated cows (29.2 % vs 13.5 %, respectively). Although pregnancy rates varied for cows exposed to different environmental temperatures, there was no significant effect of temperature on pregnancy rates. Temperatures were elevated throughout the experimental period.

Recipient pregnancy rates were influenced by the developmental stage and quality of embryos transferred (Table

Table 6-4. Pregnancy rates of embryo recipient and artificially inseminated lactating dairy cows, determined by milk progesterone concentrations (Day 21 post-estrus) and rectal palpation (Day 40 to 60 post-estrus), as influenced by mean maximum environmental temperature during embryonic development (Days 0 to 7 post-embryo transfer or insemination.

Environmental Temperature (C)	N	Pregnancy rate %					
		Embryo transfer			Insemination		
		Day 21	Day 40	(n)	Day 21	Day 40	(n)
Overall**	637	47.6	29.2	(113)	18.0	13.5	(524)
< 32 C	350	35.5	22.9	(48)	15.6	13.9	(302)
> 32 C	287	54.7	33.8	(65)	20.7	13.1	(222)

** X^2 = 13.562, P<0.001, Day 21 pregnancy rate different between embryo transfer and artificial insemination.

** X^2 = 16.674, P<0.001, Day 40 to 60 pregnancy rate different between embryo transfer and artificial insemination

6-5). They were higher (44.0 %, $P<0.05$) when early blastocyst stage embryos were transferred to recipients as compared to pregnancy rates following transfer of either advanced blastocysts or any of the morula stage embryos. Pregnancy rates decreased ($P<0.05$) as the quality of transferred embryos decreased and were 14.5 % lower following transfer of fair compared to excellent quality embryos.

The effect of synchrony between donor and recipient at the time of embryo transfer on subsequent pregnancy rates of recipients is presented in Table 6-6. Pregnancy rates were highest (38.5 %) when embryos were transferred to recipients displaying estrus at the same time (synchrony = 0) as donors. Recipient pregnancy rates decreased ($P<0.01$) as the degree of estrus asynchrony increased. Pregnancy rates decreased sooner and to a greater extent as the degree of estrous cycle asynchrony increased for recipients in estrus after donors compared to recipients with the same degree of relative asynchrony but in estrus prior to donors.

Discussion

Overall superovulatory response of donor heifers, ova fertilization rate and the percentage of embryos of transferable quality were consistent with those in previously published reports which utilized donors of similar breed types and parity, and similar gonadotrophin treatments

Table 6-5. Recipient pregnancy rates (day 40) following transfer of embryos of various developmental stages and quality scores.

Group	N	(%)	Pregnancy rate (%)
Embryo total	113		29.2
EMBRYO STAGE[a]			
Morula	34	30.1	29.4
Advanced morula	20	17.7	15.0
Early blastocyst	25	22.1	44.0
Advanced blastocyst	34	30.1	26.5
EMBRYO QUALITY[b]			
Excellent	73	64.6	34.5
Good	29	25.7	26.6
Fair	10	8.8	20.0
Poor	1	0.9	0.0

[a] $X^2 = 12.36$ $P<0.05$, pregnancy rate different among embryo stages of development.

[b] $X^2 = 14.72$ $P<0.05$, pregnancy rate different among embryo quality scores.

Table 6-6. Recipient pregnancy rates[a] (day 40) as influenced by the degree of estrous cycle synchronization between recipient and donor cattle.

Synchrony (h)		N	Pregnancy rate (%)
− 24	Recipient estrus	8	12.5
− 12	before donor	6	33.3
0		52	36.5
+ 12	Recipient estrus	21	19.1
+ 24	after donor	26	23.1

[a] $X^2 = 11.62$, $P<0.05$, pregnancy rate different among synchrony groups.

(Putney et al., 1986; Gordon et al., 1987; Monty and Racowsky, 1987).

Embryos recovered from heifers and subjected to morphological evaluation were considered generally to be at the expected developmental stage (morula to early blastocyst) for Day 7 of pregnancy. A high degree of variability was observed in embryo morphological development and quality within and among heifers. Embryos recovered from superovulated cattle often display a wide range of developmental stages (Newcomb, 1982; Putney et al., 1986). Variability in stage of embryo development may be due, partially, to the extended time period during which ovulation and fertilization may occur in superovulated cattle (Maxwell et al., 1978, Angel, 1979; Yadav et al., 1985).

Although summer heat stress conditions did not appear to affect superovulatory response of dairy heifers, increasing maximum environmental ambient temperatures, particularly those greater than 32 C, increased the incidence of poorer quality and retarded embryos recovered from heifers. Similar detrimental effects of maternal heat stress on subsequent embryo development and quality have been observed (Gordon et al., 1987; Monty and Racowsky, 1987; Putney et al., 1986, Chapter 3).

Recipient pregnancy rates increased as the quality of embryos transferred increased, with excellent embryos yielding the highest pregnancy rates. Pregnancy rates were lower when morulae were transferred to recipients and increased as the developmental stage of the embryo progressed to the blastocyst stage. Similar effects of embryo grade and stage of development on subsequent recipient pregnancy rates have been reported (Wright 1981; Schneider et al., 1980; Hasler et al., 1987; Putney et al., 1987, Chapter 5). In the present study pregnancy rates were higher when recipients were in estrus 0 to 12 h before the donor. This apparent advantage to the recipient, that is in estrus prior to the donor, has been observed previously (Halley et al., 1979; Hasler et al., 1987; Putney et., 1987, Chapter 5). It has been suggested (Schneider et al., 1980; Seidel, 1981; Hasler et al., 1987) that embryos recovered from superovulated cattle may be slightly advanced in development relative to those from nonsuperovulated animals.

Pregnancy rates of lactating dairy cows to artificial insemination ranged from 15.6 to 20.7 % during stressful months of the year when mean maximum ambient temperatures ranged from 31.7 to 34.9 C. Similar decreases in reproductive performance of lactating dairy cattle exposed to summer heat stress conditions have been reported (Gwazdauskas et al., 1973; Badinga et al., 1985). Decreased reproductive performance of lactating dairy cattle is probably due to an inability to maintain a normal body temperature under heat stress conditions because of a high rate of internal heat production associated with milk production. Increased temperature of the oviduct and uterus, may adversely affect embryo viability and lead to increased embryonic death. For example, elevated rectal and uterine temperatures during the first few days after insemination are correlated with increases in abnormal embryos (Putney et al., 1986) and depressions in pregnancy rates of dairy cattle (Gwazdauskas et al., 1973).

In the present study, pregnancy rates of lactating cows were increased significantly when embryos were transferred to such cows at Day 7 post estrus. Pregnancy rates at Day 21 post estrus were 29.6 % higher for recipient cows, and this advantage decreased to 15.7 % by Days 45 to 60 post-estrus. Increased pregnancy rates of recipient cows indicates that the early bovine embryo of cows that are artificially inseminated is extremely sensitive to harmful effects of maternal hyperthermia and fails to survive to Day 21 post estrus. Embryo transfer may bypass this early developmental period of extreme embryonic thermal sensitivity. However, bovine embryos continue to be sensitive to maternal hyperthermia beyond the period of early cleavage and blastocyst formation (Biggers et al., 1987). Heat stress during the post transfer period of blastocyst elongation and maintenance of the corpus luteum (Days 8 to 21 post estrus) may increase the rate of pre- and post-implantation embryonic mortality. This is supported by an 18.4 % decrease in recipient pregnancy rates between Days 21 and 60 post estrus.

However, embryonic thermal sensitivity after Day 7 of development does not appear to be as great as during the earlier period before Day 7. Fetal losses between Days 21 and 60 post estrus were two to three times higher for embryo recipient (18.4 %) compared to artificially inseminated (4.5 %) cows. Similar estimates of fetal losses after Day 25 post estrus with nonsurgical embryo transfer (17 to 20 % loss) have been reported (Sreenan, 1976; Christie et al., 1980). These are approximately double those observed with artificial insemination (Helman and Lamming, 1979).

In summary, summer heat stress of superovulated Holstein heifers increased the incidence of retarded embryos (fewer than 16 cells) as well as embryos graded as fair to poor in quality. Pregnancy rates of lactating Holstein embryo recipient cows were significantly higher than those of cows presented for artificial insemination. Embryo transfer may provide an alternative to artificial insemination to circumvent heat stress-induced infertility in cattle. For example, groups of donor cattle could be superovulated during hot weather and managed under environmental shade systems until embryo recovery. Good quality embryos could then be transferred at Day 7, to recipients that are not as sensitive to heat stress e.g., non-lactating recipients or recipients that are environmentally protected, at a time when embryos appear to be less sensitive to heat stress. Additionally, embryo cryopreservation and subsequent embryo transfer may be a means of bypassing the early period of thermal sensitivity. Sensitivity of bovine embryos to heat stress between Days 7 to 17 post estrus may contribute to decreased pregnancy rate of recipient cows between Days 21 and 40 to 60 of pregnancy. Therefore, it is important to determine if bovine embryos continue to be sensitive to heat stress beyond the first week of development.

CHAPTER 7

HEAT STRESS INDUCED ALTERATIONS IN THE SYNTHESIS AND SECRETION OF PROTEINS AND PROSTAGLANDINS BY CULTURED CONCEPTUSES AND UTERINE ENDOMETRIUM

Introduction

The bovine embryo is extremely sensitive to maternal heat stress. Intermittent thermal stress from 30 h after the onset of estrus until Day 7 of pregnancy increased the incidence of abnormal or retarded embryos recovered from dairy heifers (Putney et al., 1988, Chapter 3). Bovine blastocysts continue to be sensitive to heat stress beyond the first week of development. Maternal heat stress between Days 8 and 16 after insemination reduced conceptus weight and caused a trend towards increased pregnancy failure in beef cattle (Biggers et al., 1987).

The cause of heat stress-induced embryonic mortality is not known. Embryonic mortality in hyperthermic cows may be due, in part, to thermal-induced alterations in gene expression and synthesis of conceptus proteins involved in embryonic development and maternal recognition of pregnancy. Since heat stress retards embryonic development (Putney et al., 1988), it is possible that embryonic mortality may result from failure of embryos to produce biochemical signals at the proper time, such as for preventing CL regression.

Maintenance of CL function and suppression of uterine luteolytic signals are required for the conceptus to survive and pregnancy to persist in cattle (Lukaszewska and Hansel, 1980; Kindahl et al., 1976). The pre-attachment conceptus secretes an array of proteins that are involved in maintenance of the CL and continuation of pregnancy (Northey and French, 1980; Betteridge et al., 1984; Bartol et al., 1985; Knickerbocker et al., 1986a). These antiluteolytic proteins may act locally on the gravid uterine horn to alter the synthesis and release of luteolytic $PGF_{2}\alpha$ from the endometrium by stimulating the production of an endometrial inhibitor of prostaglandin synthesis (Gross et al., 1988a).

Uterine secretions are believed to provide nutrients essential for embryonic development during early pregnancy in many domestic species including cattle (Bazer, 1975). Alterations in the secretory activity of the uterine endometrium in response to severe hyperthermia may alter embryonic development and contribute to hyperthermia-induced pregnancy failure.

Little information is available on the effects of heat stress on the biochemical processes within the bovine conceptus and endometrium. This study examined whether heat stress, in vitro, alters protein and prostaglandin synthesis and secretion by conceptuses and endometrium obtained on Day 17 of pregnancy to determine whether heat stress-induced alterations in these functions of conceptus and endometrial tissues might play a role in embryonic mortality.

Materials and Methods

Collection of Conceptuses and Uterine Endometrium

Beef cattle (Angus or Brangus) were utilized for collection of bovine conceptuses and endometrium. Cattle were observed for estrous behavior and bred by natural service to Angus bulls. Animals were slaughtered on Day 17 after estrus and reproductive tracts were recovered. Conceptuses were flushed from uteri with ~50 ml Eagle's minimal essential medium (MEM, Gibco, Grand Island, NY), according to procedures described by Helmer et al. (1987) Conceptuses were weighed, placed in fresh MEM and cultured as described below.

Following flushing of the conceptus, the uterine horn ipsilateral to the corpus luteum was opened longitudinally along the antimesometrial border and endometrial slices excised. Intercaruncular endometrium was dissected free from myometrial tissue, blotted on sterile gauze, weighed and cultured in MEM.

In Vitro Culture

Preparation of a modified Eagle's minimum essential medium (MEM) and supplies for tissue culture were as described by Godkin et al. (1982) except that medium was additionally supplemented with 1 % (v/v) MEM vitamin mix

(Gibco). Conceptuses and endometrium were transferred to sterile plastic petri dishes and cultured as described by Basha et al. (1979). Endometrial explants (500 mg; ~2-3 mm^3) or whole Day 17 conceptuses were cultured in 20 ml of MEM supplemented with 100 uCi L-[4,5-^{3}H]leucine (specific activity [SA] = ~150 Ci/mmol, Amersham Corporation, Arlington Heights, IL) and 200 g arachidonic acid (Sigma Chemical Company, St. Louis, MO) under an atmosphere of 47.5 % O_2, 50 % N_2 and 2.5 % CO_2 (v/v/v). Cultures were maintained in the dark on rocking platforms. Control thermoneutral cultures were maintained at 39 C for 24 h. Heat-stressed cultures were acclimated at 39 C for 6 h and then placed at 43 C for 18 h. Culture medium from both treatment groups was sampled (1 ml) at 0, 3, 6, 9, 12, 18 and 24 h after initiation of culture. Samples of medium were stored at -70 C until assayed for incorporation of [^{3}H]leucine into protein and concentrations of prostaglandins.

Preparation of Culture Medium and Tissue for Analysis

At termination of culture, endometrial and conceptus tissues were isolated from culture medium by centrifugation (3500 X g, 4 C, 30 min) and decanting of supernatant. Tissues were homogenized in 50 mM Tris-acetate buffer (8 ml, pH 7.5) which contained 1 mM phenylmethylsulfonylflouride, 1 mM ethylenediamine tetraacetic acid and 2 % (v/v) Nonidet P-40. Homogenates were stored at -70 C until used for subsequent analyses. To remove low molecular weight compounds and unincorporated radiolabeled precursors, culture medium recovered at the end of incubation was dialyzed extensively (three changes of 4 liters) against deionized-distilled water using Spectrapor dialysis tubing (Spectrum Medical, Los Angeles, CA) with a 6000 to 8000 molecular weight exclusion limit.

Protein Determination

Incorporation of [^{3}H]leucine into secreted and intracellular proteins was determined by trichloroacetic acid (TCA) precipitation. Samples (50 ul) of solubilized tissue and conditioned culture medium were placed onto Whatman 3MM paper (Whatman, Clifton, NJ), previously saturated with 20 % TCA (w/v), and allowed to dry. Precipitation of proteins onto filter paper and removal of nonproteinaceous compounds was accomplished by serial washings of the filter paper with 20 % TCA, 5 % TCA and 95 % ethanol as described by Mans and Novelli (1961). Radioactivity of precipitated protein was determined by scintillation spectrometry.

Electrophoresis

One-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (1D SDS-PAGE) was perfomed using the buffer system of Laemmli (1970). Separation of proteins was by electrophoresis in 12.5 % (w/v) polyacrylamide gels. Two-dimensional (2D) SDS-PAGE was performed according to a modification of the method described by Roberts et al. (1984). Samples were dissolved in 0.01 ml of 5 mM K_2CO_3 containing 9.3 M urea, 2 % (v/v) Nonidet P-40 and 0.5 % (w/v) dithiothreitol, and resolved in the first dimension by isoelectric focusing in 4 % (w/v) acrylamide tube gels containing N,N'-diallyltartdiamide, 8.0 M urea, 2 % (v/v) Nonidet P-40 and 5.1 % (v/v) ampholines (pI. 3-10, 5-7 and 9-11; 50:36:16 by volume, respectively, Serva, Heidelberg, FGR). First dimension gels were equilibrated in 0.07 M Tris-HCl buffer (pH 6.8) containing 1 % (w/v) SDS and 1 % (v/v) 2-mercaptoethanol and subjected to electrophoresis in the second dimension in 12.5 % (w/v) ployacrylamide slab gels according to the procedure of Laemmli (1970). Proteins were localized by Coomassie Blue R-250 staining and fluorography as described by Roberts et al. (1984). Fluorographs were prepared with sodium salicylate as a fluor and Kodak XAR film.

Iodination of Protein A

Protein A (Genzyme, Boston, MA) was iodinated by a modification of the procedure of Markwell and Fox (1978). Briefly, 20 μg IODO-GEN (Pierce Chemical Company, Rockford, IL) was added to 375 ul of 0.02 M KPO_4 (pH 7.0) containing 0.4 M NaCl. Next, 20 ug Protein A and 5 ul of carrier free Na[^{125}I] (500 uCi, SA = ~ 16.9 Ci/ug of I, Amersham Corporation) were added to the reaction tube and incubated for 20 min. Unreacted ^{125}I was separated from radioiodinated Protein A by chromatography on a Pharmacia PD-10 column

(Pharmacia, Piscataway, NJ) using 1 % gelatin in phosphate buffered saline as the eluent.

Western Blotting

Conceptus proteins present in culture supernatants were resolved (1D SDS-PAGE) under reducing conditions on 12.5 % polyacrylamide gels. Slab gels were equilibrated for 15 min in 25 mM Tris-HCl buffer (pH 6.8) containing 200 mM glycine and 20 % (v/v) methanol, overlayed with a nitrocellulose membrane (BA85, 0.45 µm, Schleicher and Schuell, Keene, NH) and subjected to electrophoresis (200 mA, for 24 h at 4 C) toward the cathode. Following electrophoretic transfer, nitrocellulose membranes were stained with amido black 10B (Research Organics, Cleveland, OH) (Harper et al., 1986) and immunoblotted. Nonspecific binding of proteins to nitrocellulose was blocked with 10 mM Tris (pH 7.6) containing 3 % (w/v) gelatin, 0.8 % (w/v) NaCl and 0.05 % (v/v) Tween 20. Blocked membranes were incubated (2 h at room temperature) with rabbit antiserum to ovine trophoblast protein-1 (anti-oTP-1, F.W. Bazer, University of Florida) which exhibits binding to bovine trophoblast protein-1 (bTP-1) (Helmer et al., 1987) or with normal rabbit serum at dilutions of 1:100 in incubation buffer (10 mM Tris-HCl, pH 7.6, containing 1 % [w/v] gelatin, 0.8 % [w/v] NaCl and 0.05 % [v/v] Tween 20). Following incubation, membranes were washed (30 min) in incubation buffer to remove unbound antiserum and further incubated (2 h at room temperature) with ^{125}I-labeled Protein

A (10^6 cpm/ml). Membranes were rinsed with distilled H_2O, washed (24 h at 4 C) with Tris-HCl (pH 7.6), dried and visualized by autoradiography to detect Protein A-antibody-antigen complexes bound on the nitrocellulose membrane.

Quantification of Proteins Separated by PAGE

Conceptus secretory proteins present in culture medium were resolved (1D SDS-PAGE) under reducing conditions on 12.5 % gels. Individual lanes, representing separate conceptus cultures, were isolated and sequentially sectioned into 2 mm slices to generate a profile of radioactive proteins. Slices were individually solubilized by incubation in 0.4 ml H_2O_2 for 2 h at 70 C, mixed with scintillation fluid and radioactivity determined by scintillation spectrometry.

Measurement of Prostaglandins

Conceptus and endometrial conditioned culture medium was analyzed for $PGF_2\alpha$ using a radioimmunoassay (RIA) procedure (Knickerbocker et al., 1986b) modified to use an antibody characterized by Kennedy (1985). Radioisotopes $[5,6,8,11,12,-14,15-^3H]PGF_2\alpha$ (SA = ~160-180 Ci/mmole) and $[5,6,8,12,14,15-^3H]PGE_2\alpha$ (SA = ~140-170 Ci/mmole) were purchased from Amersham Corporation (Arlington Heights, IL). Standard curves were prepared in MEM with known amounts of radioinert $PGF_2\alpha$ (10-5000 pg, Sigma Chemical Company). An antiserum dilution of 1:5000 was utilized with a minimum sensitivity of 25 pg per tube. Cross-reactivities of the $PGF_2\alpha$ antiserum with other prostaglandins were 94 % for

$PGF_1\alpha$, 2-4 % for PGE_2 and <0.1 % for PGFM, PGE_1 and arachidonic acid. Due to the high cross-reactivity with $PGF_2\alpha$, $PGF_2\alpha$ measurements are defined as PGF. Unextracted samples (300 ul) from tissue incubations contained [^{3}H]leucine (approx. 25,000 cpm). To correct for this, samples were assayed with and without the addition of [^{3}H]$PGF_2\alpha$. Following charcoal-dextran isolation of bound material, the bound radioactivity for duplicate samples without [^{3}H]$PGF_2\alpha$ were subtracted from values for duplicate samples which contained [^{3}H]$PGF_2\alpha$. An inhibition curve containing $PGF_2\alpha$ (5 ng/ml) was assayed serially in 25, 50, 100, 200 and 300 ul volumes (final volume of 300 ul with blank MEM) with [^{3}H]leucine (approx. 25,000 cpm). This inhibition curve (corrected for [^{3}H]leucine background) was parallel to the standard curve, with the test for homogeneity of regression indicating that the curves did not differ. Inter- and intra-assay coefficients of variation were 17.7 and 12.9 %, respectively.

Samples were also analyzed for PGE_2 with an RIA procedure (Lewis et al., 1978) modified to analyze unextracted MEM and to use an antibody characterized by Lewis et al. (1978). Standard curves were prepared in MEM with known amounts of radioinert PGE_2 (10-5000 pg, Sigma Chemical Company). An antiserum dilution of 1:6000 was used with a minimum sensitivity of 25 pg per tube. Cross-reactivities of the PGE_2 antiserum with other prostaglandins were 24 % for

PGE_1, 1.7 % for $PGF_{2\alpha}$ and <0.1 % for PGFM, PGF_1 and arachidonic acid. Correction for nonspecific binding due to the presence of [^{3}H]leucine in samples was done as described for the PGP RIA. An inhibition curve containing PGE_2 (5 ng/ml) was assayed serially in 25, 50, 100, 200 and 300 ul volumes (final volume of 300 ul with blank MEM) with [^{3}H]leucine (approx. 25,000 cpm). This inhibition curve was parallel to the standard curve, with the test for homogeneity of regression indicating that the curves did not differ. Inter- and intra-assay coefficients of variation were 18.2 and 14.1 %, respectively.

Statistical Analyses

Effect of treatment on conceptus and endometrial secretory activity was analyzed by least-squares analysis of variance utilizing the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Analysis of conceptus data was conducted with a nested design with conceptuses nested within treatment. The statistical model was $Y_{ijk} = \text{treatment}_i + \text{time}_j + \text{conceptus}_k(\text{treatment}_i) + \text{treatment}_i{*}\text{time}_j + \text{residual}$. Endometrial data was modeled with cow cross-classified across treatment groups. The statistical model was $Y_{ijk} = \text{treatment}_i + \text{time}_j + \text{cow}_k + \text{treatment}_i{*}\text{time}_j + \text{residual}$. Protein and prostaglandin secretion rates were characterized by polynomial regressions. Tests for homogeneity of regressions were used to detect differences in secretion rates due to treatment.

Results

Quantitative Protein Synthesis and Secretion

Secretion of proteins by tissues was determined by measuring incorporation of [^{3}H]leucine into TCA-precipitable proteins released into culture medium. Conceptus and endometrial tissues remained viable throughout the duration of culture as suggested by linear accumulation of newly synthesized, [^{3}H]leucine-labeled proteins secreted into culture medium (Figure 7-1). Regression analysis indicated that protein secretion rates varied over the duration of culture according to tissue type (endometrium < conceptus) and temperature treatment. Prior to initiation of heat stress, secretion of proteins by tissues within heat stress treatment groups was similar to that of thermoneutral control tissues (conceptus: 4888 ± 3363 vs 7654 ± 3684; endometrium: 1161 ± 154 vs 1135 ± 154 dpm/mg tissue/6 h). Elevation of incubation temperature from 39 to 43 C reduced conceptus radiolabeled protein synthetic capacity, resulting in a 54.2 % decrease (P<0.01) in incorporation of [^{3}H]leucine into secretory proteins and a 66.8 % decrease (P<0.01) in incorporation into conceptus tissue proteins at the end of culture (Table 7-1). In contrast, heat stress of endometrium did not affect the rate of incorporation of [^{3}H]leucine into either secretory or tissue proteins.

Table 7-1. Incorporations of [^{3}H]leucine into TCA-precipitable protein in tissue and medium collected after 24 h of culture by conceptuses and endometrium cultured at thermoneutral (39 C) or heat stress (43 C) temperatures (Least squares mean ± sem).

Tissue Type X Treatment	N	Tissue wet wt	Radiolabeled protein (DPM/mg tissue/24 h)	
		(mg)	secreted	tissue
Endometrium				
Thermoneutral	10	500	8767 ± 322	73405 ± 4197
Heat stress	10	500	8266 ± 322	79514 ± 4197
Conceptus				
Thermoneutral	5	81.2 ± 12.3	64431 ± 3656*	167498 ± 27247*
Heat stress	6	64.8 ± 8.2	29487 ± 3656	55529 ± 27247

* P<0.01, for thermoneutral vs heat stress for conceptuses.

Qualitative Analysis of Proteins

Qualitative differences in [^{3}H]leucine incorporation into proteins synthesized by conceptus and endometrial tissues were evaluated. Electrophoretic examination (2D SDS-PAGE) of radiolabeled tissue proteins revealed a complex spectrum of newly-synthesized, radiolabeled proteins. Representative fluorographs of protein patterns presented in Figure 7-2 are typical of all tissue samples analyzed. Heat stress altered the array of proteins present in conceptus and endometrial tissues. In particular, heat stress caused conceptuses to synthesize proteins with apparent molecular weights of 70,000 (pI 6.2) and 91,000 (pI 5.6). These heat shock proteins were also apparent in low levels in tissues from endometrial cultures maintained at 39 C and were present in more abundant amounts in endometrial tissues from cultures incubated at 43 C.

Conditioned medium from the conceptus and endometrial cultures were analyzed by 1D SDS-PAGE and radiolabeled proteins localized by fluorography (Figures 7-3 and 7-4). The predominant radiolabeled polypeptides detected in conceptus culture supernatants appeared as three bands having apparent molecular weights of 22,000, 24,500 and 26,000, these proteins appeared to be bTP-1. The predominant proteins secreted by endometrium were of M_r = 29,000, 38,000 and 50,000. Heat stress did not appear to alter the spectrum

Figure [illegible]-1. Incorporation of [^{3}H]leucine into polypeptides released into culture medium during 24 h of culture by endometrial and conceptus tissues. Proteins present in culture medium (50 ul) were TCA precipitated onto filter paper and [^{3}H]leucine labeled proteins measured by scintillation spectrometry. Shown are least squares means and the best fit (conceptus: r=.89,P<0.001; endometrium: r=.94, P<0.001) regression line calculated by multiple regression (_________ = thermoneutral; __ _ __ _ = heat shock).

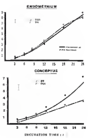

ENDOMETRIUM
DPM (x10³) ³H protein/mg TISSUE
p .001
r .94
3
6
9
12
15
18
21
24
CONCEPTUS
DPM (x10³) ³H protein/mg TISSUE
r .89
p .001
1
2
3
4
5
6
7
3
6
9
12
15
18
21
24
INCUBATION TIME

Figure 7-2 Two dimensional polyacrylamide gel electrophoresis of proteins present in endometrial and conceptus tissues. Samples were endometrium and conceptuses cultured at 39 C (thermoneutral) or 43 C (heat shock). Proteins were separated in the first dimension by isoelectric focusing and in the second dimension by SDS PAGE using 12.5 % (w/v) polyacrylamide gels. Radiolabeled proteins were localized by fluorography. Equal amounts of radioactivity (200,000 dpm) were loaded on each gel. Note the predominant heat-induced proteins present in heat-stressed tissues were absent or reduced in thermoneutral tissues (1 = 70,000 pI 6.2, 2 = 91,000 pI 5.6).

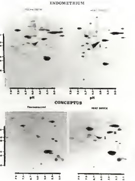
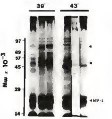

ENDOMETRIUM
THERMONEUTRAL
HEAT SHOCK
Mw x 10 3
100
60
50
29
20
16
1
pH
CONCEPTUS
Thermoneutral
HEAT SHOCK
Mw x 10 3
2
1
pH

Figure 7-3 One dimensional polyacrylamide gel electrophoresis of secretory proteins present in conceptus culture medium. Samples were medium supernatants of conceptuses collected after 24 h of culture. After dialysis (M_r cutoff = 60 to 80 kDa), proteins were separated by SDS-PAGE using 12.5 % (w/v) polyacrylamide gels. Radiolabeled proteins were localized by fluorography. Equal volumes (100 ul) of medium were loaded per lane. Fluorographic exposure time differed for conceptuses (42 > 39 C) in order to examine qualitative differences in radiolabeled polypeptides in medium between treatments. Note that the major [^{3}H]leucine-labeled proteins identified in conceptus secretions were reduced by heat stress (arrows).


39°
43°
97
69
57
45
29
14
Mw x 10^{-3}
◄
◄
◄ bTP-1

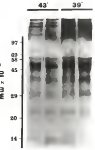

43°
39°
Mw x 10^{-3}
97
69
58
45
29
20
14

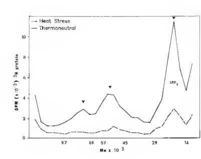
Figure 7-4. One dimensional polyacrylamide gel electrophoresis of secretory proteins present in endometrial culture medium. Samples were medium supernatants of endometrium collected after 24 h of culture. After dialysis (M_r cutoff = 6000 to 8000), proteins were separated by SDS-PAGE using 12.5 % (w/v) polyacrylamide gels. Radiolabeled proteins were localized by fluorography. Equal volumes (100 ul) of medium were loaded per lane.

of radiolabeled proteins secreted into culture medium by conceptuses or endometrium.

Quantitative Analysis of Proteins

To quantitatively evaluate the relative radiolabeling of secretory protein species present in culture medium, equal volumes of conceptus-conditioned medium from each conceptus were electrophoretically separated (1D SDS-PAGE) and the resolved proteins solubilized from the gel matrix in sequential 2 mm slices and counted for radioactivity. Profiles of radioactivity were similar among conceptuses within the same treatment group and hence were averaged to examine treatment effects (Figure 7-5). The predominant radiolabeled polypeptides (representing 27.5 % of total radioactivity) appeared as a single peak with an apparent molecular weight range of 22,000 to 26,000. This protein peak appears similar in molecular weight to bovine trophoblast protein-1 (bTP-1) previously characterized by Helmer et al. (1987). Overall, heat stress of conceptuses resulted in a 63.2 % reduction (P<0.01) in the total quantity of electrophoretically resolved radiolabeled proteins detected relative to control tissues (94,789 ± 2758 vs 29,064 ± 683 dpm/mg tissue/24 h). Relative labeling of secretory protein species with apparent molecular weight ranges of aproximately 50,000 and 85,000 were decreased 76.3 and 76.8 %, respectively. Conceptus secretory proteins migrating coincident with bTP-1 were reduced 71.7 % (P<0.01) relative

Figure 7-5. Electrophoretic profile of [^{3}H]leucine-labeled polypeptides accumulated into conceptus culture medium after 24 h of culture. After dialysis (M_r cutoff = 6000 to 8000), proteins were separated by 1D SDS-PAGE using 12.5 % (w/v) polyacrylamide gels and solubilized from the slab matrix with H_2O_2. Equal volumes (100 ul) of conceptus conditioned medium from each conceptus were loaded per lane. Shown are mean profiles of radiolabeled proteins (dpm/ mg tissue) for thermoneutral (______) and heat-stressed (- - - -) conceptuses. Note that peaks in detected radioactivity, corresponding to predominant proteins identified by 1D SDS-PAGE and fluorography, were reduced (P<0.05) by heat stress (arrows).



Heat Stress
Thermoneutral
DPM (x 10^-3) 3H protein
0
2
4
6
8
10
97
69
57
45
29
14
Mw x 10^3
bTP1

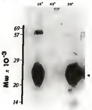
to proteins obtained from thermoneutral conceptuses (7,381 ± 698 vs 26,099 ± 2758 dpm/mg tissue/24 h).

Immunoblotting

As a final test of whether bTP-1 secretion was reduced by heat stress, antiserum to oTP-1, an immunologically related species (Helmer et al., 1987), was used to detect bTP-1 in culture medium by immunoblotting. Autoradiograms of immunoblots are depicted in Figure 7-6. Visual apprasial of autoradiograms indicated that oTP-1 antiserum bound specifically to protein species with an apparent molecular weight range of 22 to 26,000. These antibody-reactive proteins were of similar molecular weight to those in the predominant protein bands detected by fluorography (Figure 7-3) as well as those in the major radioactive peak solubilized from 1D SDS-PAGE gels (Figure 7-5). Collectively, these different experimental approaches confirm that proteins within this molecular weight range may in part represent the bTP-1 component of conceptus secretory proteins. Heat stress of the conceptus resulted in a marked reduction in secretory proteins transfered to nitrocellulose that bound oTP-1 antiserum, as indicated by a decrease in [^{125}I]Protein A labeling of antibody-bound proteins detected by autoradiography. Minor cross-reactivity of antiserum with a polypeptide species of 66,000 molecular weight was detected. This protein most likely represents bovine serum albumin present in conceptus cultures. No detectable

Figure 7-6. Immunoblotting of bTP-1 released into conceptus culture medium during 24 h of culture. After dialysis (M_r cutoff = 6 to 8 kDa), proteins were separated by 1D SDS-PAGE using 12.5% (w/v) polyacrylamide gels, electrophoretically transfered to nitrocellulose membrane, and immunoblotted with either nonimmune (not shown) or anti-oTP-1 rabbit serum. Equal volumes (100 ul) of conceptus-conditioned medium from each conceptus were loaded per lane. Antibody-protein complexes bound to nitrocellulose were detected by ^{125}I-Protein A labeling of antibody and autoradiography. Note specific binding of oTP-1 antibody to bTP-1 is reduced by heat stress (arrow)

CONCEPTUS WESTERN BLOT



antibody-protein complexes were observed on autoradiograms of nitrocellulose blots incubated with non-immune rabbit serum.

Prostaglandin Secretion

In order to examine the effect of heat stress on prostaglandin synthetic rate of conceptus and endometrial tissues, samples of medium were analyzed for PGF and PGE content via radioimmunoassay. It has previously been shown that most PGF and PGE present in culture medium of endometrial explants is derived from de novo synthesis of PG during culture (Thatcher et al., 1984). Endometrium maintained at 39 C synthesized and released prostaglandins primarily during the first 3 h of incubation (Figure 7-7). Concentrations of PGE detected in culture medium of thermoneutral endometrium increased throughout culture resulting in higher (P<0.01) levels of PGE than PGF (Table 7-2) at the end of culture. Prior to heat stress, secretion of prostaglandins by endometrial tissues was similar to that of thermoneutral control tissues (heat stress vs thermoneutral, PGF= 3.4 ± 0.5 vs 2.8 ± 0.5; PGE = 2.5 ± 0.5 vs 4.1 ± 1.5 pg/ml/mg tissue/6 h). Elevation of incubation temperature from 39 to 43 C stimulated endometrial PGF release, resulting in a 1255 % increase (P<0.01) in concentrations detected in culture medium. Concentrations of PGE were not affected by heat stress. As a result, heat-stressed endometrium secreted more (P<0.01) PGF than PGE .

Figure 7-7. Secretion of prostaglandins (PGF and PGE$_2$) into culture medium by endometrium and conceptuses. Samples of conditioned medium (300 l) were analyzed for prostaglandins by radioimmunoassay. Shown are profiles of PGF and PGE$_2$ radiolabeled for thermoneutral (_____) and heat-stressed (_ _ _ _) endometrium and conceptuses. Endometrial secretion of PGF ($P<0.001$) and conceptus secretion of PGE$_2$ ($P<0.05$) increased in response to heat stress.

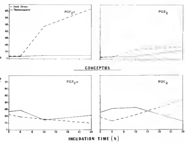

ENDOMETRIUM
Heat Stress
Thermoneutral
PGF$_2$α
PGE$_2$
CONCEPTUS
PGF$_2$α
PGE$_2$
Pg / ml / mg Tissue
0
10
20
30
40
50
60
70
3
6
9
12
15
18
21
24
INCUBATION TIME (h)

Table 7-2 Concentrations of PGF and PGE in medium supernatants collected after 24 h of culture of conceptuses and endometrium at thermoneutral or heat stress temperatures (least squares mean ± sem).

Tissue Type X Treatment		Prostaglandin (pg/ml/mg tissue/24h) PGF	Prostaglandin (pg/ml/mg tissue/24h) PGE
Endometrium			
Thermoneutral	10	5.6 ± 0.7	13.1 ± 2.9
Heat stress	10	74.2 ± 17.9**	18.6 ± 5.4
Conceptus			
Thermoneutral	5	21.5 ± 8.8	12.2 ± 4.1
Heat stress	6	9.6 ± 3.3	43.9 ± 33.8*

** P<0.001, for thermoneutral vs heat stress for endometrium.

* P<0.05, for thermoneutral vs heat stress for conceptuses. Due to a large coefficient of variation data were transformed to Log prior to analysis.

Although conceptuses released prostaglandins during culture, there was a trend towards decreased prostaglandin concentrations in medium as the duration of culture increased (Figure 7-7). Imposition of heat stress, however, stimulated conceptus production of PGE_2, resulting in a 360 % increase ($P<0.05$) in concentrations detected in culture medium. Concentrations of PGF were not affected by heat stress.

Discussion

Physiological studies have demonstrated that the preimplantation embryo is extremely sensitive to high environmental temperature and humidity (Alliston and Ulberg, 1961; Elliott et al., 1968; Elliott and Ulberg, 1971; Ulberg and Sheenan, 1973; Putney et al., 1986). Although embryos are most sensitive to harmful effects of high temperature during initial stages of cleavage (Dutt, 1963; Alliston et al., 1965), the developing blastocyst continues to be sensitive to heat stress beyond the first week of development. The present study has shown that in vitro elevation of incubation temperature of Day 17 bovine conceptuses from 39 to 43 C reduces total protein synthetic capacity while enhancing the synthesis of heat shock or stress proteins (hsp). Similar effects of in vivo thermal stress on subsequent in vitro protein production by conceptuses have been noted in swine (Wetteman et al., 1984)

These in vitro data suggest that high environmental temperature may severely alter conceptus metabolic activity in vivo and lead to reduced growth rates and failure of conceptuses to produce biochemical signals in adequate amounts required for preventing CL regression.

It has been reported that maternal heat stress in cattle (Biggers et al., 1987) and swine (Wettemann et al., 1984) between Days 8 and 16 after insemination reduces conceptus and CL weights at the time of conceptus recovery on Day 17. Smaller conceptuses may not develop the biosynthetic capacity required to properly signal the maternal system to maintain CL function, as evidenced by findings that heat stress caused reduced CL weight and a trend towards increased pregnancy failure (Wettemann et al., 1984; Biggers et al., 1987).

In the present study, conceptuses secreted several radiolabeled polypeptides into culture medium. Culture medium was enriched in a group of low molecular weight proteins (20 to 26 kDa). Other smaller and larger molecular weight protein species were detected by fluography, but they were not the major secretory products. These conceptus secretory proteins were similar in molecular weights and relative proportions to those described previously (Bartol et al., 1985). Antiserum to oTP-1 crossreacts immunologically with several components of the array of low molecular weight proteins present in culture medium. This complex of proteins is referred to as bovine trophoblast protein-1 complex (bTP-1) and is believed to be involved in preventing luteal regression during early pregnancy (Helmer et al., 1987). Heat stress, by altering total protein synthetic capacity of conceptuses, induced a marked reduction in secretory proteins, particularly proteins within the bTP-1 complex. This may compromise successful rescue of the corpus luteum from regression since this event may depend directly upon the appropriate timing and quantity of secretory proteins produced by the conceptus. For example, pregnancy rates are higher after transfer of two embryos compared to transfer of a single embryo into bovine recipients (Heyman et al., 1984). Further, trophoblastic vesicles prepared from Day 14 bovine conceptuses and transferred into cyclic cows will maintain CL function for 25 to 37 days (Heyman et al., 1984) as well as increase pregnancy rates when transferred with blastocysts into recipient cattle (Heyman et al., 1987).

Concentrations of prostaglandins released by tissues maintained at 39 C were similar to those synthesized in vitro by bovine endometrium from Day 17 of pregnancy (Thatcher et al., 1984). Heat stress of endometrial tissues resulted in a marked increase in release of PGF into culture medium. Increased PGF synthetic capacity of heat-stressed endometrium may be due to alterations in membranes resulting in increased mobilization of substrates, such as arachidonic acid, for prostaglandin synthesis (Flint et al., 1986). Similar increases in endometrial PGF production in response to heat stress have been reported in vivo. Heat stress of gilts increased concentrations of 13,14-dihydro-15-keto PGF (PGFM), a metabolite of $PGF_{2}\alpha$, in the peripheral circulation and altered luteal function as indicated by reduced plasma concentrations of progesterone (Wetteman et al., 1984; Hoagland and Wetteman, 1984). Similar increases in peripheral concentrations of PGFM in response to heat stress have been reported in postpartum cattle (Lewis et al., 1984). Since maintenance of luteal function in cattle is associated with alterations in endometrial prostaglandin production (Thatcher et al., 1984; Gross et al., 1988b), increased endometrial prostaglandin secretion in response to thermal stress may compromise CL function and initiate partial or complete luteal regression.

Conceptuses maintained at a high incubation temperature synthesized proteins not synthesized by control tissues. The predominant heat shock protein (hsp) was of 70,000 Da, similar to that found in other heat-shocked mammalian tissues (Nover, 1984). Thus, response of bovine conceptuses to thermal stress, i.e., the enhanced synthesis of hsps, is similar to the heat shock response observed in a wide range of organisms (Nover, 1984). Considerable research has focused on the identification and characterization of heat shock proteins. The most prominent of these proteins, the mammalian 70 kDa hsp, is synthesized by mouse (Wittig et al., 1983) rat (Mirkes, 1987) and rabbit (Heikkila and Schultz,

1984) embryos maintained at high incubation temperatures. While of uncertain function, it has been assumed that hsps may play an essential role in cellular homeostasis and thermotolerance during periods of environmental stress (Loomis and Wheeler, 1980; Li and Werb, 1982).

Elevation of incubation temperature of endometrial explants did not appear to alter protein synthetic rate of tissues. However, endometrial tissues in both treatment groups synthesized proteins similar in molecular weight (Mr = 70,000 and 91,000) to hsps identified in heat-stressed conceptuses. The intensity of these endometrial proteins was enhanced in tissues exposed to heat stress culture conditions. The trauma of tissue slicing or the incubation conditions of the present experiment may have resulted in diminished protein synthesis as well as the expression of hsps by endometrium in both treatment groups. Similarly, Hightower and White (1981) reported that several high molecular weight stress-induced proteins were synthesized by sliced mammalian tissues in vitro but not synthesized by tissues in vivo.

In summary, the effects of heat stress on protein and prostaglandin synthetic rate of the Day 17 conceptus and pregnant endometrium was examined in vitro. Elevation in tissue incubation temperature from 39 to 43 C induced a dramatic reduction in conceptus protein synthesis and secretion and stimulated release of PGF by pregnant endometrium. These in vitro results suggest that exposure of pregnant cows to high environmental temperature and humidity, as often occurs during summer months of the year, may disrupt the balance between conceptus and endometrial biochemical factors responsible for maintenance of pregnancy. Therefore, it is important to further characterize the modulation of uterine prostaglandin biosynthesis by pregnant and nonpregnant cows in response to both in vitro and in vivo heat stress.

CHAPTER 8

PROSTAGLANDIN SECRETION BY ENDOMETRIUM OF PREGNANT AND CYCLIC CATTLE AT DAY 17 AFTER ESTRUS IN RESPONSE TO IN VITRO HEAT STRESS

Introduction

In vitro heat stress of conceptuses recovered at Day 17 of pregnancy altered embryonic gene expression and induced a marked decrease in the sythesis and secretion of proteins into culture medium (Putney et al., 1987, Chapter 7). Since heat stress retards development and reduces protein synthetic capacity of conceptuses, it is possible that embryonic mortality may result from failure of conceptuses to produce adequate amounts of antiluteolytic biochemical signals necessary to attenuate the sequence of events leading to luteolysis (Bartol et al., 1985; Knickerbocker et al., 1986a) and maintain a functional corpus luteum (CL).

There is considerable evidence that the uterine luteolysin in cattle is prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (Lauderdale, 1974; Thatcher et al., 1984). Exogenous administration of $PGF_2\alpha$ will induce luteolysis (Lauderdale, 1974; Thatcher et al., 1976) while passive immunization against $PGF_2\alpha$ extends corpus luteum lifespan (Fairclough et al., 1981). Furthermore, increased $PGF_2\alpha$ concentrations have been demonstrated in uterine flushings (Bartol et al., 1981), uterine venous plasma (Nancarrow et al., 1973) and in endometrial tissues (Thatcher et al., 1984) prior to luteolysis.

Heat stress of endometrium recovered at Day 17 of pregnancy induced a marked increase in release of $PGF_{2}\alpha$ into culture medium (Putney et al., 1987; Chapter 6). Similar increases in endometrial $PGF_{2}\alpha$ production in response to heat stress has been observed in vivo. Thermal stress between Days 8 and 16 after insemination increased 13,14-dihydro-15-keto $PGF_{2}\alpha$ (PGFM), a metabolite of $PGF_{2}\alpha$ in the peripheral circulation of gilts (Wettemann et al., 1984). Additionally, Lewis et al. (1984) reported that prepartum heat stress increased the concentrations of PGFM during the postpartum period in dairy cattle. Increased concentrations of systemic PGFM may reflect greater uterine secretion of $PGF_{2}\alpha$ in response to heat stress. The present study examined whether in vitro heat stress would increase the secretion of $PGF_{2}\alpha$ by perifused endometrial tissue from pregnant and cyclic cattle at Day 17 post-estrus. Alterations in uterine $PGF_{2}\alpha$ secretion may exert pronounced effects on CL lifespan and therefore maintenance of pregnancy.

Materials and Methods

Collection of Uterine Endometrium

Beef cattle (Angus and Brangus) were observed for behavioral estrus and either bred by natural service (n=8) or not bred (n=4) Cattle were slaughtered on Day 17 after estrus and reproductive tracts recovered and flushed with Minimal Essential Medium (MEM, 40 ml) to collect conceptus tissue and confirm pregnancy. The uterine horn ipsilateral to the CL was opened longitudinally along the antimesometrial border and endometrium dissected free from myometrium over a surface area of 6-7 cm^2. Each endometrial sample was placed into sterile Krebs-Ringer Bicarbonate solution (KRB) which was gassed with O_2:CO_2 (95:5), washed twice with fresh KRB, and dissected into three pieces of 2 cm^2 each.

In Vitro Perifusion of Endometrium

Each of the three endometrial samples were placed into a bilateral perifusion device which allows for separation of myometrial and luminal surfaces of endometrium so that each side can be perifused separately in a 0.5 ml hemi-chamber (Lacroix and Kann, 1983) Tissue was stretched and anchored into each device with the aid of four needles. Each chamber was placed into a water bath (35 C), attached to dual 60 ml syringes containing KRB, and perifused at a rate of 3 ml/10 min for 5 h using a Harvard infusion pump system (Harvard Apparatus, Dover, MA). Fractions (3 ml) were collected every 10 min for a total of 30 fractions.

Each of the three endometrial perifusion preparations within each cow were subjected to a different temperature and oxytocin ([Oxy], 1 IU/ml KRB, Sigma Chemical Company, St. Louis, MO) treatment sequence (Figure 8-1). 1) control-oxytocin: 1 h at 39 C, 2 h at 39 C, 0.5 h at 39 C with oxytocin, 0.5 h at 39 C and 1 h at 39 C, 2) heat-oxytocin: 1 h at 39 C, 2 h at 42 C, 0.5 h at 42 C with oxytocin, 0.5 h at 42 C and 1 h at 39 C; 3) heat-KRB: 1 h at 39 C, 2 h at 42 C, 0.5 h at 42 C with no oxytocin, 0.5 h at 42 C and 1 h at 39 C. Temperature treatments were imposed on endometrium by placing perifusion devices in water baths maintained at either 39 C or 42 C and perifusion of tissues with KRB maintained at either 39 C or 42 C.

Measurement of $PGF_2\alpha$

Fractions were analyzed for $PGF_2\alpha$ using a radioimmunoassay (RIA) procedure (Knickerbocker et al., 1986b) modified to use an antibody characterized by Kennedy (1985) and validated by Gross et al. (1988b). Radioinert $PGF_2\alpha$ were purchased from Sigma Chemical Company and radiolabeled [5,6,8,9,11,12,14,15-^{3}H]$PGF_2\alpha$ (specific activity of ~160-180 µCi/mmole) was purchased from Amersham Corporation (Arlington Heights, IL). Cross-reactivities of the $PGF_2\alpha$ antiserum with other prostaglandins were 94 % for $PGF_1\alpha$, 2.4 % for PGE_2 and < 0.1 % for PGFM, PGE_1 and arachidonic acid. Due to the high cross-reactivity with PGF_1 , $PGF_2\alpha$ measurements are defined

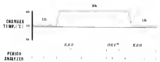


Figure 8-1. Schematic representation of the perifusion temperature and oxytocin (1 IU/ ml KRB) treatment sequence imposed on endometrium from cyclic and pregnant cows. Treatments were 1) Control-oxytocin. 1 h at 39 C, 2 h at 39 C, 0.5 h at 39 C with oxytocin, 0.5 h at 39 C and 1 h at 39 C, 2) Heat-oxytocin. 1 h at 39 C, 2 h at 42 C, 0.5 h at 42 C with oxytocin, 0.5 h at 42 C and 1 h at 39 C; 3) Heat-KRB 1 h at 39 C, 2 h at 42 C, 0.5 h at 42 C, 0.5 h at 42 C and 1 h at 39 C.

as PGF. Inter- and intra-assay coefficients of variation were 12.2 % and 9.7 %, respectively.

Statistical Analysis

Data were analyzed by Least Squares Analysis of Variance using the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Prostaglandin secretion rates of luminal, myometrial or luminal + myometrial sides of endometrium were analyzed statistically for the following perifusion treatment periods: (1) 1 h pre-heat stress, (2) 2 h pre-oxytocin, (3) 0.5 h with or without oxytocin, (4) 0.5 h post-oxytocin, and (5) 1 h post-heat stress (Fig. 1). Sources of variation for statistical analyses were: Y_{ijklm} = $status_i$ (pregnant and cyclic) + $status_i[cow_j]$ + $treatment_k$ + $status_i*treatment_k$ + $status_i[cow_j]^*treatment_k$ + $side_l$ (luminal and myometrial) + $treatment_k*side_l$ + $status_i*side_l$ + $status_i[cow_k]*side_l$ + $status_i*treatment_k*side_l$ + $status_i[cow_k]*treatment_k*side_l$ + $time_m$ + $treatment_k*time_m$ + $side_l*time_m$ + $status_i*time_m$ + $treatment_k*side_l*time_m$ + $status_i*treatment_k*time_m$ + $status_i*side_l*time_m$ + $status_i*treatment_k*side_l*time_m$ + residual. The statistical model and analyses for total (luminal + myometrial sides of endometrium) PGF_2 secretion are presented in Table 8-1.

Table 8-1. Least Squares Analyses of Variance of total (luminal + myometrial surfaces) endometrial PGF secretion rates for thermoneutral vs heat stress (period 2; 2 h at either 39 or 42 C) and oxytocin (period 3; 0.5 h at either 39 with oxytocin, or 42 C with or without oxytocin) treatments.

	Perifusion treatment periods			
	2		3	
Sources of Variation	df	MS x 10^4	df	MS x 10^4
Status[a]	1	2487.9*	1	1827.0**
Cow(status)	7	448.2**	7	146.9**
Treatment[b]	2	1957.8**	2	776.9*
Status x Treatment	2	6.4	2	32.4
Cow(status) x Treatment	10	16.6	10	176.0
Time[c]	11	104.1**	2	48.3
Status x Time	11	27.2*	2	16.5
Treatment x Time	22	30.1**	4	30.8
Status x Treatment x Time	22	15.1	3	35.4
Residual	162	13.1	22	37.2

[a] Status of endometrium: cyclic vs pregnant

[b] Perifusion at either 39 C + oxytocin, 42 C + oxytocin or 42 C without oxytocin

[c] Perifusion, 10 min time periods

* P< 0.05, ** P< 0.01

Results

Endometrial tissues remained viable throughout perifusion as indicated by sustained PGF release into KRB solution throughout the duration of perifusion (Figure 8-2). Mean secretion rates of total (luminal + myometrial surfaces) PGF when KRB was perifused across tissues maintained at thermoneutral temperatures (treatment period 1) were 46 % lower (P< 0.01) for endometrium from pregnant compared to that of cyclic cows (678 ± 34 vs 1282 ± 128 pg/10 min; Table 8-2).

Endometrial secretion of PGF occurred primarly from the luminal surface of tissues for each reproductive status, but the magnitude of the difference between sides was less (P< 0.01) for pregnant (181 pg/10 min) than for cyclic (482 pg/10 min) cows (Tables 8-3 and 8-4). Mean PGF secretion rates by tissues maintained at thermoneutral temperature (treatment period 1) were 77 % higher (P< 0.05) for luminal compared to myometrial surfaces of endometrium from pregnant cows (427 ± 14 vs 241 ± 19 pg/10 min) and 120 % higher (P< 0.01) for cyclic cows (884 ± 82 vs 402 ± 38 pg/10 min). Although myometrial surfaces secreted less PGF than luminal surfaces, prostaglandin secretion responses to temperature and oxytocin treatments were parallel between sides (Tables 8-3 and 8-4). Therefore, PGF responses detected in luminal and myometrial KRB perfusates were

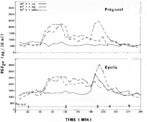


Figure 8-2. Total (luminal + myometrial surfaces) secretion rate (pg/ 10 min KRS) of PGF$_{2\alpha}$ by endometrium of (a) pregnant and (b) cyclic cows perifused at 39 C with an oxytocin challenge (______) or 42 C with (__ __ __) or without (- - - -) an oxytocin challenge. Perifusion periods analyzed were: (1) 1 h at 39 C, (2) 2 h at either 39 or 42 C, (3) 0.5 h at either 39 with oxytocin or 42 C with (OXY) or without (saline) oxytocin, (4) 0.5 h at either 39 or 42 C, and (5) 1 h at 39 C.

Table 8-2. Total (luminal + myometrial surfaces) endometrial secretion rates (mean ± se pg/10 min) of PGF into KRB during perifusion at the following treatment periods: (1) 1 h at 39 C, (2) 2 h at either 39 or 42 C, (3) 0.5 h at either 39 with oxytocin or 42 C with or without (KRB) oxytocin, (4) 0.5 h at either 39 or 42 C, and (5) 1 h at 39 C.

Groups	Perifusion treatment period				
	1^a	2^b	3^b	$4^{a,c}$	5
Pregnant:					
39 -OXY	857 ± 87	609 ± 33	530 ± 43	541 ± 54	505 ± 50
42 -OXY	618 ± 43	1572 ± 96	2300 ± 265	1005 ± 153	581 ± 51
42 -KRB	560 ± 34	1465 ± 114	870 ± 87	865 ± 67	523 ± 35
Cyclic:					
39 -OXY	1337 ± 146	1323 ± 100	2352 ± 300	1463 ± 159	1166 ± 86
42 -OXY	1316 ± 156	2323 ± 153	3024 ± 344	1731 ± 177	1104 ± 107
42 -KRB	1193 ± 105	2124 ± 165	2040 ± 261	1595 ± 128	1199 ± 69

[a] P< 0.01 for status and cow(status)
[b] See table 1 for statistical analyses and differences
[c] P< 0.01 for treatment

Table 6-3 Secretion rates (mean + se pg/10 min) of PGF into KRB by luminal surfaces of endometrium perifused during various treatment periods[a]

Groups	Perifusion treatment period				
	1[b]	2[c]	3[d]	4[e]	5[f]
Pregnant:					
39 -OXY	478 ± 42	386 ± 18	343 ± 33	362 ± 35	337 ± 22
42 -OXY	435 ± 29	1080 ± 55	1332 ± 97	613 ± 67	391 ± 31
42 -KRB	354 ± 32	869 ± 69	563 ± 77	537 ± 58	327 ± 31
Cyclic:					
39 OXY	896 ± 101	955 ± 62	1594 ± 222	1088 ± 118	879 ± 77
42 -OXY	948 ± 93	1410 ± 78	1952 ± 127	1144 ± 83	914 ± 93
42 -KRB	809 ± 78	1362 ± 99	973 ± 81	1043 ± 129	907 ± 78

[a] See Table 2 and text for description of periods analyzed
[b] P< 0.01 for status and cow(status)
[c] P< 0.05 for status and P< 0.01 for cow(status), treatment, cow(status)*treatment, time and treatment*time
[d] P< 0.01 for status and treatment and P<0.05 for cow(status)
[e] P< 0.01 for status, cow(status) and treatment
[f] P< 0.01 for status and cow(status)

Table 8-4. Secretion rates (mean ± se pg/10 min) of PGF into KRB by myometrial surfaces of endometrium perifused during various treatment periods[a].

Groups	Perifusion treatment period				
	1[b]	2[c]	3[d]	4[e]	5[f]
Pregnant.					
39 -OXY	325 ± 43	223 ± 15	232 ± 15	213 ± 21	200 ± 15
42 -OXY	192 ± 14	494 ± 41	883 ± 151	404 ± 88	205 ± 11
42 -KRB	208 ± 18	597 ± 45	351 ± 51	337 ± 56	203 ± 12
Cyclic:					
39 -OXY	434 ± 47	368 ± 17	800 ± 101	393 ± 51	304 ± 14
42 -OXY	417 ± 83	914 ± 76	1172 ± 199	683 ± 108	381 ± 32
42 -KRB	356 ± 37	783 ± 88	960 ± 148	389 ± 35	293 ± 33

[a] See table 2 and text for description of periods analyzed
[b] $P < 0.01$ for status and cow(status)
[c] $P < 0.05$ for status, time and status*time and $P < 0.01$ for cow(status), treatment and cow(status)*treatment
[d] $P < 0.01$ for status, cow(status) and treatment
[e] $P < 0.05$ for status and $P < 0.01$ cow(status) and treatment
[f] $P < 0.01$ for status and $P < 0.05$ for cow(status)

combined to assess total PGF secretion rates by endometrium in response to heat stress and oxytocin challenge (Figure 8-2; Tables 8-1 and 8-2).

Prior to initiation of heat stress (treatment period 1), PGF secretion rates by endometria within each reproductive status were similar among treatment groups (Table 8-2). Elevation of perifusion temperature from 39 to 42 C stimulated prostaglandin secretory capacity of endometrium regardless of reproductive status (Table 8-2). Overall secretion rates of PGF during the first 2 h of heat stress (treatment period 2) were 68 % greater (P< 0.01) for cyclic (2223 ± 96 vs 1323 ± 100 pg/ 10 min) and 149 % greater (P< 0.01) for pregnant (1518 ± 65 vs 609 ± 33 pg/ 10 min) endometrium. Heat stress induced a rapid (within 10 min) release of PGF by endometrium from both pregnant and cyclic cows However the response was not sustained by pregnant endometrium, and declined precipitously within 1 h after initiation of thermal stress (Figure 8-2).

Perifusion of KRB supplemented with oxytocin (treatment period 3) induced a rapid 164 % increase (P< 0.01) in secretion of PGF by heat-stressed pregnant endometrium (2300 ± 265 vs 870 ± 87 pg/ 10 min). In contrast, pregnant endometrium perifused at 39 C did not respond to oxytocin treatment with increased PGF release (Table 8-2). Cyclic endometrium, regardless of perifusion temperature, increased the rate of PGF secretion in response to oxytocin challenge;

a 48 % increase ($P< 0.05$) in PGF secretion by heat-stressed tissues (3024 ± 344 vs 2040 ± 261 pg/ 10 min) and a 77 % increase ($P< 0.05$) by thermoneutral tissues (2353 ± 300 vs 1323 ± 100 pg/ 10 min) was detected. At the conclusion of oxytocin perifusion, secretion rates of PGF declined rapidly and returned to pre-oxytocin rates of secretion within 30 min of perfusion with KRB (treatment period 4; Table 8-2, Figure 8-2).

At the conclusion of heat stress, a reduction in incubation temperature from 42 to 39 C (treatment period 5) reduced ($P< 0.01$) endometrial prostaglandin secretion rates. Mean overall secretion rates of PGF into KRB by tissues within each reproductive status were similar among treatment groups in period 5 (Figure 8-2, Table 8-2).

Discussion

Establishment of pregnancy in cattle is dependent upon interactions between antiluteolytic and luteolytic factors produced by the periattachment conceptus and uterine endometrium, respectively. Maintenance of luteal function in pregnant cattle is associated with diminished secretion of PGF_2^{α} during the expected period of luteolysis (Thatcher et al., 1984; Gross et al., 1988b). Likewise, the release of PGF_2^{α} from the uterus in response to estrogen (Thatcher et al., 1984) and oxytocin (Lafrance & Goff, 1985) is attenuated during early pregnancy. Data from the present study using endometrial perifusions confirm that endometrial secretion of $PGF_{2\alpha}$ is reduced during early pregnancy in cattle.

Heat stress induced a rapid increase in endometrial secretion rates of PGF by luminal and myometrial surfaces for pregnant and cyclic cows. Increased PGF synthetic capacity of endometrium exposed to heat stress may be due to heat induced alterations in endometrial cellular membranes resulting in increased mobilization of substrate for prostaglandin biosynthesis. The primary cellular site for the action of heat damage on tissues is membranes (Bowler et al., 1973; Hahn, 1982). Exposure to high ambient temperature is accompanied by alterations in membrane lipid composition (Anderson & Parker, 1982) as well as increases in membrane fluidity, phospholipase activity and phosphoinositide turnover (Calderwood et al., 1987). Heat-induced increases in the turnover of membrane phospholipids and release of fatty acids, such as arachidonic acid, may provide substrates for prostaglandin synthesis (Flint et al., 1986). Arachidonic acid, the principle substrate for $PGF_{2\alpha}$ synthesis, is present in large quantities within the bovine endometrium prior to the time of increased endometrial $PGF_{2\alpha}$ production and luteolysis (Hansel et al., 1975; Shemesh & Hansel, 1975). Furthermore, exogenous arachidonic acid will stimulate prostaglandin production by pregnant and cyclic endometrial tissues in vitro (Thatcher et al., 1984). Elevated secretion rate of PGF by heat-stressed pregnant endometrium was not sustained throughout the duration of heat stress perifusion, but declined precipitously within 1 h after initiation of thermal stress. This decrease in PGF secretion may be due, in part, to a depletion of the intracellular pool of arachidonic acid in endometrial cells of heat-stressed pregnant cows. Perifusion of heat-stressed endometrium of two pregnant cows with KRB supplemented with arachidonic acid (50 ug/ml KRB) resulted in sustained increases in PGF release throughout the period of heat stress (unpublished data, D.J Putney, 1988).

Similar increases in uterine PGF$_{2}\alpha$ secretion in response to heat stress in vitro by endometrial explants from cows at Day 17 of pregnancy have been reported (Putney et al., 1987). Furthermore, in vivo heat stress of gilts between Days 8 to 16 of pregnancy increased 13,14-dihydro-15-keto PGF$_{2}\alpha$ (PGFM), a metabolite of PGF$_{2}\alpha$, in the peripheral circulation and compromised luteal function as indicated by reduced plasma concentrations of progesterone during Days 13 through 19 (Hoagland & Wettemann, 1984; Wettemann et al., 1984). In cattle, prepartum heat stress increases peripheral concentrations of PGFM and reduces CL diameter post partum (Lewis et al., 1984). Decreased concentrations of progesterone in plasma of gilts and CL size in cattle may be due to heat-induced partial regression of the CL in stressed animals, which may be attributed to greater uterine secretion of $PGF_2\alpha$ (reflected by greater concentrations of systemic PGFM) in heat stressed than in thermoneutral animals. Since maintenance of luteal function in cattle is associated with decreased endometrial $PGF_2\alpha$ secretion, it is likely that increases in endometrial prostaglandin secretion rate in response to heat stress may compromise CL function, initiate partial or complete luteal regression and contribute to pregnancy failure.

Endometrium from pregnant cattle, when perifused at thermoneutral temperature, was unable to respond to oxytocin with an increased secretion of PGF, whereas endometria from cyclic cattle responded with an increase in the secretion of PGF. These in vitro perifusion data are in agreement with previous in vivo and in vitro data which demonstrate an attenuated increase in secretion of prostaglandins in response to oxytocin during early pregnancy in cattle (Lafrance & Goff, 1985; Gross et al., 1988b). However, oxytocin induced a rapid increase in luminal and myometrial secretion rates of PGF by endometrium from heat-stressed pregnant cows as well as tissues of cyclic cows, regardless of perifusion temperature. Such a rapid induction of endometrial prostaglandin secretion by oxytocin has been noted previously in vivo for cyclic cattle (Oyedipe et al.,
1984; Lafrance & Goff, 1985). In vitro, heat stress perturbated the physiological difference between responses of pregnant and cyclic endometrial tissues to oxytocin.

Oxytocin-induced secretion rates of $PGF_{2}\alpha$ for pregnant endometrium exposed to heat stress were similar to those by cyclic endometrium perifused at thermoneutral temperatures.

A heat stress induced responsiveness of pregnant endometrium to oxytocin may be due in part to increased availability of oxytocin receptors because of alterations in membrane fluidity, or to activation of intracellular signal transduction associated with oxytocin binding to its receptor on the endometrial cell membrane. In particular, oxytocin has been reported to stimulate endometrial prostaglandin synthesis by increasing the hydrolysis of membrane bound phosphoinositides to diacylglycerol and inositol phosphates with subsequent release of arachidonic acid from diacylglycerol (Flint et al., 1986). Similarly, heat stress has been reported to stimulate membrane phospholipase activity and phosphoinositide turnover (Calderwood et al., 1987) as well as activating cAMP production (Calderwood et al., 1985). Hence, heat stress may potentiate oxytocin induced prostaglandin synthesis by activating oxytocin receptor mediated second messengers such as inositol phosphates and diacylglycerol.

Alternatively, heat stress may potentiate oxytocin induced synthesis of endometrial $PGF_{2}\alpha$ by altering factors which regulate prostaglandin synthesis during early pregnancy. One possibility is that heat stress alters activity of an endogenous intracellular endometrial inhibitor of prostaglandin synthesis, the activity of which is normally increased during early pregnancy (Basu & Kindahl, 1987; Gross et al., 1988a). A heat stress induced increase in $PGF_2\alpha$ secretion of pregnant endometrium in response to oxytocin may be a consequence of a decrease in the activity of the prostaglandin inhibitor. Regardless of the mechanism, the sequence of events leading to luteolysis and termination of pregnancy may be initiated by heat stress.

In summary, bilateral perifusion devices were utilized to measure PGF secretion by luminal and myometrial surfaces of bovine endometrium in response to in vitro heat stress. When perifused at thermoneutral temperatures (39 C), endometrial secretion rates of PGF by pregnant cattle were lower than those of cyclic cattle, and tissues from pregnant cows were unable to respond to oxytocin with an increased rate of secretion of PGF. Heat stress (42 C) induced a rapid increase in PGF secretion regardless of reproductive status and induced a responsiveness of pregnant endometrium to an oxytocin challenge. Increased PGF secretion rates and responsiveness to oxytocin by endometrium from pregnant cows in response to heat stress suggests an ability of this stressor to compromise anti-luteolytic mechanisms (inhibition of $PGF_2\alpha$ synthesis) that are normally initiated by the conceptus during early pregnancy. These in vitro results support the concept that heat stress may disrupt the finely tuned balance between antiluteolytic and luteolytic factors produced by the conceptus-endometrial units that are responsible for establishment of pregnancy. However, in vivo experiments with heat-stressed pregnant cows are required to test this hypothesis.

CHAPTER 9

MODULATION OF UTERINE PROSTAGLANDIN BIOSYNTHESIS BY PREGNANT AND NONPREGNANT COWS AT DAY 17 POST ESTRUS IN RESPONSE TO IN VIVO AND IN VITRO HEAT STRESS

Introduction

Exposure of pregnant cattle to increased ambient temperature may adversely affect conceptus development due to heat-induced alterations in function of the reproductive tract and endocrine system of the dam. Establishment of pregnancy in cattle is dependent upon maintenance of luteal function and is associated with diminished secretion of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) during the expected period of luteolysis (Thatcher et al., 1984, 1986; Gross et al., 1988a). Likewise, release of $PGF_2\alpha$ from the uterus in response to estrogen (Thatcher et al., 1984) and oxytocin (Lafrance & Goff, 1985) is attenuated during early pregnancy. Heat stress perturbs this physiological difference in prostaglandin secretion between cyclic and pregnant cattle. For example, increases in uterine $PGF_2\alpha$ secretion by endometrial explants from cows at Day 17 of pregnancy, in response to heat stress in vitro, have been reported (Putney et al., 1987, 1988a, Chapters 7 and 8). Oxytocin-induced secretion rates of $PGF_2\alpha$ were increased from perifused pregnant endometrium exposed to heat stress and similar to those by cyclic endometrium maintained at thermoneutral temperatures.

Heat stress may increase synthesis of endometrial $PGF_{2\alpha}$ by altering factors which regulate prostaglandin biosynthesis during early pregnancy. One possibility is that heat stress alters activity of an endogenous intracellular endometrial, prostaglandin synthesis inhibitor (EPSI), the activity of which is normally increased during early pregnancy (Basu and Kindahl, 1987; Gross et al., 1988a). A heat-stress induced increase in $PGF_{2\alpha}$ secretion may be a consequence of a decrease in the activity of the prostaglandin inhibitor.

Since maintenance of luteal function in cattle is associated with decreased endometrial $PGF_{2\alpha}$ secretion, it is likely that increases in endometrial prostaglandin secretion, in response to heat stress, may compromise CL function and contribute to pregnancy failure when cattle are subjected to high ambient temperatures. This study examined whether acute summer heat stress of pregnant and non-pregnant dairy cows altered uterine prostaglandin secretion in vivo, and whether heat stress of endometrium from pregnant and cyclic cows alters the activity of EPSI.

Materials and Methods

Experiment I

Environmental treatments

Lactating dairy cows (n=30) were observed twice daily for behavioral estrus and artificially inseminated with frozen semen between 4 and 14 hours following detection of estrus. Cows were managed under routine conditions with a shade management system (Roman-Ponce et al., 1977) to protect animals from daily heat stress. On Day 17 post insemination, cows were assigned randomly to either thermoneutral, or heat stress environmental treatments. Control cows (n=15) were maintained in a thermoneutral environment of 25.4 ± 2.2 C in an evaporatively cooled barn. Heat-stressed cows (n=15) were exposed for 7.5 h (beginning at 0800 h) to summer heat stress in which maximum environmental ambient temperatures were 35.0 ± 2.0 C. Respiration rates and rectal temperatures of all experimental cows were monitored prior to cannulation and oxytocin challenge and at the end of environmental treatments.

Collection of peripheral blood samples

Prior to imposition of treatments (0700 h, on Day 17 post insemination) cows were cannulated with V/8 polyvinyl catheters (Bolab Incorporated, Lake Havasu City, AZ) via the jugular vein. Blood samples (10 ml) were collected, via a syringe, into heparinized glass tubes at 1 h intervals during the first 5 h (0800 to 1300 h) and then at 15 min intervals during the subsequent 1 h (1300 to 1400 h) of environmental treatments. Oxytocin (100 IU, Vedco Company, Arcadia, CA) was then administered via the jugular catheter at 1400 h, and samples of blood were collected at 5 min post oxytocin injection and then at 15 min intervals from 1415 to 1530 h of environmental treatments. Blood samples were placed immediately on ice and centrifuged at 10,000 x g for 20 min within 30 min of collection. Plasma was stored at -20 C until assayed for 13,14-dihydro-15-keto $PGF_2\alpha$ (PGFM) and progesterone (P_4). Progesterone concentrations were measured in plasma samples (1 sample/cow) collected at the time of, or, within 1 h after cannulation, prior to imposition of environmental treatment.

Recovery of conceptuses

Immediately following environmental treatments and the last blood sample, both groups of cows were maintained at thermoneutrality (24 ± 2 C) in the evaporatively cooled barn, and conceptuses recovered nonsurgically to confirm pregnancy status. Conceptuses were flushed from the uterine lumen with Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY) by intermittent gravity flow through a 2-way, round tip Foley catheter (16 to 20 French gauge, American Hospital Supply, Jacksonville, FL) introduced into the uterus. The flushing effluent was collected into a flat bottom grid searching dish (Fisher Scientific, Orlando, FL), and conceptuses were isolated and weighed. Cows from which no embryonic tissue was recovered were considered to be nonpregnant.

Measurement of plasma PGFM concentrations

Unextracted plasma samples (300 ul) were assayed for PGFM using a polyethylene glycol radioimmunoassay (RIA) procedure and antibody characterized by (Eley et al., 1981; Guilbault et al., 1984). Radiolabeled 13,14-dihydro-15-keto-[5,6,8,9,11,12,14-^{3}H] PGF$_{2}$α (specific activity of ~ 157 Ci/mmole) was purchased from Amersham Corporation (Arlington Heights, IL). Inter- and intra-assay coefficients of variation were 4.0 % and 14.0 %, respectively. Minimum sensitivity of the RIA was 30 pg PGFM/ml plasma.

Measurement of plasma progesterone concentrations

Plasma samples were analyzed for P$_4$ using a validated RIA procedure and antibody characterized by Knickerbocker et al., 1986a). Radiolabeled [1,2,6,7-^{3}H]progesterone (specific activity of ~ 97 Ci/mmole) was purchased from New England Nuclear (Boston, MA). Cross-reactivity of the progesterone antibody was < 1% with 17α-hydroxyprogesterone, 20β-hydroxyprogesterone, 20α-hydroxy-progesterone, cortisol, testosterone androstenedione and estradiol-17 . A standard curve was prepared by adding known amounts of radioinert P$_4$ (0-500 pg/ml; Sigma Chemical Company) to phosphate-buffered saline (pH 7.4) containing 1 g gelatin/l (PBSg). An antiserum dilution of 1:40000 was used with a minimum sensitivity of 15 pg per tube. Progesterone from plasma samples (200 ul) was extracted in duplicate with freshly distilled benzene:hexane (2 ml, 1:2 v/v). Solvent containing the extracted P_4 was dried under air and brought to a 500 ul assay volume with the addition of PBSg. Intra-assay coefficients of variation was 10.1%.

Experiment II

Collection of uterine endometrium

Beef cattle were observed for behavioral estrus and either bred by natural service (n=5) or not bred (n=5). Cattle were slaughtered on Day 17 after estrus and reproductive tracts recovered and flushed with Minimal Essential Medium (MEM, 50 ml; Gibco, Grand Island, NY) to collect conceptus tissue and confirm pregnancy. The uterine horn ipsilateral to the CL was opened longitudinally along the antimesometrial border, endometrium dissected free from myometrium, and endometrial slices excised. Intercaruncular endometrium was blotted on sterile gauze, weighed and cultured in MEM.

In vitro culture of endometrium

Endometrial explants (500 mg; ~2-3 mm^3) were transferred to sterile plastic petri dishes and cultured in 15 ml of MEM supplemented with 1 % (v/v) MEM vitamin mix (Gibco) and 200 g arachidonic acid (Sigma Chemical Company, St. Louis, MO) under an atmosphere of 47.5 % O_2, 50 % N_2 and 2.5 % CO_2 (v/v/v). Cultures were maintained in the dark on rocking platforms. Endometrial explants from each cow were cultured at thermoneutral (39 C) and heat stress (42 C) temperatures for 8 h. Culture medium from both treatment groups was sampled (1 ml) after 8 h of culture. Samples of medium were stored at -70 C until assayed for concentrations of PGF and PGE_2 via RIA.

Preparation of the cotyledonary-prostaglandin-generating system

Parturition was induced in dairy cattle by injecting 20 mg dexamethasone (im.; Schering Corp., NJ) at 5 days prior to expected calving. Placentomes were collected within 1 h following parturition and placed into sterile saline. Fetal cotyledonary villous tissues were isolated and stored at -70 C until further analysis. Tissue (20 g/ 40 ml) was added to 0.05 M Tris-buffered 0.25 M sucrose, pH 7.4, and homogenized. Homogenates were centrifuged (800 x g) for 15 min at 4 C and then filtered through three layers of cheesecloth to remove tissue fragments. The filtrate was centrifuged (8000 x g) for 15 min at 4 C to pellet nuclei and mitochondria. Supernatants were centrifuged (100,000 x g) for 60 min at 4 C to yield a high speed supernatant and a microsomal preparation (Wlodawar and Samuelson, 1973). The microsomes were resuspended in 0.1 M potassium phosphate, pH 7.5 (20 ml) and stored in 5 ml aliquots at -70 C.

Preparation of endometrial tissues for analysis

At termination of culture, endometrial tissues were isolated from medium by centrifugation (3500 X g, 4 C, 30 min) and decanting of supernatant. Tissues were added to 0.05 M Tris-buffered 0.25 M sucrose (500 mg/ 5 ml), pH 7.4, homogenized, and then subjected to differential centrifugation as described for the cotyledonary tissue to yield a microsomal fraction and a high-speed cytosolic supernatant. The cytosolic high-speed supernatants were each stored in 5 ml aliquots at -70 C.

Assay for the inhibition of prostaglandin synthesis

Cotyledonary microsomes (0.5 ml; 500 mg tissue equivalent) were incubated in 0.1 M potassium phosphate, pH 7.5 (final incubation volume of 2 ml) with and without endometrial high-speed supernatant (0.5 ml; 250 mg tissue equivalent) and 100 g arachidonic acid (0.1 ml). Incubations were conducted at 39 C on a rocker platform for 1 h and then terminated with the addition of 0.25 ml ethanol. Each sample was centrifuged (1500 x g) for 20 min at 4 C to pellet the ethanol-precipitated material. Supernatants were analyzed for PGF via RIA.

Measurement of PGF and PGE$_2$ concentrations

Fractions from the prostaglandin inhibitor assay and media samples were analyzed for PGF$_2$ using a RIA procedure (Knickerbocker et al., 1986b) modified to use an antibody characterized by Kennedy (1985) and validated by Gross et al. (1988b). Unextracted samples from the inhibitor assay and conditioned media samples were assayed for PGF in duplicate using 0.2 and 0.3 ml aliquots, respectively. Radiolabeled

$[5,6,8,11,12,14,15\text{-}^{3}H]PGF_{2}\alpha$ (specific activity - ~160-180 Ci/mmole) was purchased from Amersham Corporation (Arlington Heights, IL). Standard curves were prepared in 0.1 M potassium phosphate, pH 7.5 for assay of fractions from the inhibitor assay and in MEM for conditioned medium samples, with known amounts of radioinert $PGF_{2}\alpha$ (10-5000 pg, Sigma Chemical Company). An antiserum dilution of 1:5000 was used with a minimum sensitivity of 25 pg per tube. Cross-reactivities of the $PGF_{2}\alpha$ antiserum with other prostaglandins were 94 % for $PGF_{1}\alpha$, 2.4 % for PGE_{2} and < 0.1 % for PGFM, PGE_{1} and arachidonic acid. Due to the high cross-reactivity with $PGF_{1}\alpha$, $PGF_{2}\alpha$ measurements are defined as PGF. Intraassay coefficients of variation was 14.4 %.

Media samples also were analyzed for PGE_{2} with an RIA procedure (Lewis et al., 1978) modified to use an antibody characterized by Lewis et al. (1978) and validated by Gross et al., 1988b. Radioisotope $[5,6,8,12,14,15\text{-}^{3}H]PGE_{2}$ (SA = ~140-170 Ci/mmole) was purchased from Amersham Corporation Standard curves were prepared in MEM with known amounts of radioinert PGE_{2} (10-5000 pg; Sigma Chemical Company). An antiserum dilution of 1:6000 was used with a minimum sensitivity of 25 pg per tube. Cross-reactivities of the PGE_{2} antiserum with other prostaglandins were 24 % for PGE_{1}, 1.7 % for $PGF_{2}\alpha$ and <0.1 % for PGFM, $PGF_{1}\alpha$ and arachidonic acid. Intra-assay coefficient of variation was 13.2 %.

Statistical Analysis

Data were analyzed by Least Squares Analysis of Variance using the General Linear Models procedure of the Statistical Analysis System (SAS, 1985). Plasma PGFM concentrations were analyzed for the pre- and post-oxytocin treatment periods seperately. Due to heterogeneity of error variance between pregnancy statuses during the post-oxytocin treatment period, PGFM data was transformed to $\log_{10}$ prior to analysis. Analyses of PGFM data were modeled with cows nested in treatment and pregnancy status. The statistical model was: Y_{ijkl} = $\text{treatment}_i + \text{status}_j + \text{treatment}_i{*}\text{status}_j + \text{cow}_k(\text{treatment}_i{*}\text{status}_j) + \text{time}_l + \text{treatment}_i{*}\text{time}_l + \text{status}_j{*}\text{time}_l + \text{treatment}_i{*}\text{status}_j{*}\text{time}_l + \text{residual}$. Concentrations of PGFM also were characterized by polynomial regressions for time trends. Tests for homogeneity of regression were utilized to detect changes in concentrations of PGFM due to environmental treatment and pregnancy status. Analysis of P_4 data were modeled with plasma sample (1 per cow) nested in pregnancy status. The statistical model was: $Y_{ij} = \text{status}_i + \text{sample}_j(\text{status}_i) + \text{residual}$. Analyses of in vitro PGF and PGE_2 data were modeled with treatment cross-classified across cows in pregnancy status groups. The statistical model was: $Y_{ijk} = \text{status}_i + \text{cow}_j(\text{status}_i) + \text{treatment}_k + \text{status}_i{*}\text{treatment}_k + \text{residual}$.

Results

Experiment I

In vivo heat stress of lactating cows

Prior to imposition of environmental treatments, mean respiration rates (58 ± 5 respirations per min) and rectal temperatures (38.8 ± .2 C) were similar among cows. Exposure to summer heat stress conditions on Day 17 post-estrus was sufficient to produce hyperthermia in heat-stressed lactating cows. Mean maximum ambient temperatures of 35.0 ± 2.0 C induced a 116 % increase in respiratory rate (134 ± 4 vs 58 ± 5 respirations per/min, P<0.001) and a 2.3 C increase in rectal temperature (41.4 ± .1 vs 38.8 ± .1 C, P<0.001) of cows managed in no-shade compared to those in the evaporatively cooled barn.

Recovery of conceptuses

Immediately following environmental treatments, conceptuses were recovered nonsurgically to confirm pregnancy status of cows. Embryos were recovered from 10 of 30 cows, resulting in an overall Day 17 pregnancy rate of 33.3 %. There was considerable variation among cows in wet weight of embryonic tissue recovered, 40 % of conceptuses (n=4) appeared severely retarded in development having lower (P<0.05) mean wet weights than conceptuses (n=6) which appeared developmentally normal (31.9 ± 6.8 vs 424.7 ± 109 mg, respectively). Retarded embryos appeared fragmented and undergoing degeneration.

Plasma progesterone concentrations

Plasma concentrations of P_4 were determined in blood samples collected immediately after cannulation and prior to imposition of environmental treatments, in order to confirm presence of an active corpus luteum in all experimental animals. Overall mean concentrations of P_4 were similar between pregnant and nonpregnant cows (11.2 ± .78 vs 10.9 ± .56 ng/ml, respectively). All cows, regardless of pregnancy status or treatment group had plasma elevated concentrations of P_4 ranging from 6.3 to 14.6 ng/ml, indicative of an active corpus luteum for Day 17 post estrus.

Prostaglandin secretion in vivo

Plasma concentrations of PGFM in the peripheral circulation of thermoneutral and heat-stressed cows, in response to environmental treatment and oxytocin challenge, are presented in Table 9-1 and Figures 9-1 through 9-4. Prior to oxytocin treatment, plasma concentrations of PGFM (overall mean of 31.8 ± 0.4 pg/ml) were similar among cows, regardless of environmental treatment or pregnancy status. An oxytocin injection induced a marked increase in overall mean PGFM concentrations during the 1.5 h post oxytocin treatment period (Table 9-1). Mean oxytocin-induced PGFM concentrations were 80 % greater (P<0.07) in nonpregnant cows and 100 % greater (P<0.01) in pregnant cows with retarded conceptuses compared to pregnant cows carrying normal conceptuses (89.1 ± 6.1 and 98.7 ± 13.6 vs 48.4 ± 4.2 pg/ml,

Table 5-1. Plasma concentrations of 15-keto-13,14-dihydro-PGF$_2$ (mean ± se pg/ml) in response to oxytocin challenge of pregnant and non-pregnant lactating dairy cattle exposed to thermoneutral or heat stress environmental treatments.

Group	N	Treatment period[a]	
		Pre-oxytocin	Post-oxytocin
Overall:			
Non-pregnant	20	32.1 ± 0.5	89.1 ± 6.1
Pregnant-retarded	4	31.5 ± 1.3	98.7 ± 13.6[b]
Pregnant-normal	6	31.2 ± 0.6	49.4 ± 4.2[b]
Non-pregnant:			
Thermoneutral	10	32.8 ± 1.0	70.7 ± 7.6[c]
Heat stress	10	31.5 ± 0.5	103.0 ± 8.6[c]
Pregnant-retarded:			
Thermoneutral	1	33.2 ± 1.0	121.0 ± 14.4
Heat stress	3	31.5 ± 1.3	90.9 ± 17.4
Pregnant-normal:			
Thermoneutral	3	32.3 ± 1.3	60.1 ± 6.3
Heat stress	3	30.0 ± 1.0	38.6 ± 4.7

[a] Pre-oxytocin (6 hours, from 0800 to 1400 h) and post-oxytocin (1.5 hours, from 1400 to 1530 h) treatment periods.

[b] P<0.07 for non-pregnant and P<0.01 for pregnant-retarded vs pregnant-normal cows during post-oxytocin period.

[c] P<0.05, for thermoneutral vs heat-stressed non-pregnant cows during post-oxytocin period.

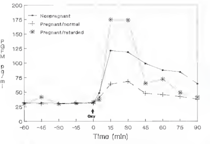


Figure 9-1 Mean concentrations of PGFM in the peripheral circulation, 60 min prior to and 90 min post oxytocin challenge (Oxy) at time = 0, of cows classified as (1) nonpregnant; (2) pregnant with normal embryos (pregnant/normal); and (3) pregnant with retarded embryos (pregnant/retarded).

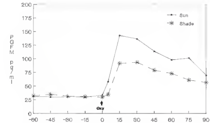


Figure 3-2. Mean concentrations of PGFM in the peripheral circulation, 60 min prior to and 90 min post oxytocin challenge (Oxy) at time = 0, of nonpregnant cows exposed to heat stress (sun) or thermoneutral (shade) environments.

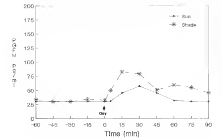


Figure 9-3 Mean concentrations of PGFM in the peripheral circulation, 60 min prior to and 90 min post oxytocin challenge (Oxy) at time = 0, of pregnant cows with normal embryos exposed to heat stress (sun) or thermoneutral (shade) environments.

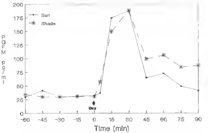


Figure 9-4. Mean concentrations of PGFM in the peripheral circulation, 60 min prior to and 90 min post oxytocin challenge (Oxy) at time = 0, of pregnant cows with retarded embryos exposed to heat stress (sun) or thermoneutral (shade) environments.

respectively; Table 9-1). Regression analysis indicated that PGFM responsiveness to oxytocin injection differed ($P<0.05$) among the three pregnancy statuses (Figure 9-1). In particular, oxytocin injection induced a rapid (within 5 to 15 min) increase ($P<0.001$) in PGFM concentrations in nonpregnant cows (32.1 ± 0.5 vs 121.4 ± 10.0 pg/ml) and pregnant cows with retarded conceptuses (31.5 ± 1.3 vs 178.2 ± 21.8 pg/ml; Figure 9-1). In contrast, pregnant cows with normal conceptuses had an attenuated response of peripheral PGFM concentrations to oxytocin challenge with an increase of only 68.3 ± 17.8 pg/ml
(Figure 9-2).

Heat stress induced a 47.1 % increase ($P<0.01$) in oxytocin-induced PGFM levels in nonpregnant cows (103.0 ± 8.6 vs 70.0 ± 7.6 pg/ml, Figure 9-2), but had no statistically significant effect on induced PGFM levels in pregnant cows with normal conceptuses (38.6 ± 4.7 vs 60.1 ± 6.3 pg/ml, a 35.8 % decrease, $P<0.10$; Figure 9-3) and pregnant cows with retarded conceptuses (90.9 ± 17.4 vs 121.0 ± 14.1 pg/ml, a 24.9 % decrease; Figure 9-4).

Experiment II

Endometrial prostaglandin synthesis in vitro

To examine the effect of heat stress on prostaglandin synthesis and secretion by endometrial tissues in vitro, samples of conditioned medium from tissues cultured at 39 or 42 C were analyzed for PGF and PGE_2 content (Table 9-2). It

Table 9-2. Mean ± sem prostaglandin (PGF and PGE_2) secretion (ng/8 h) by endometrium from pregnant and cyclic cows. Explants were incubated at 39 and 42 C for 8 h in MEM supplemented with 200 g arachidonic acid.

		Prostaglandin (ng/8 h)		EPSI*
Group	N	PGF	PGE_2	PGF (ng/h)
CYCLIC:				
39 C	5	123.4 ± 8.3^{a}	50.0 ± 3.7	$3.67 \pm .06$
42 C	5	182.7 ± 8.0^{b}	78.4 ± 4.3^{c}	$3.73 \pm .05$
PREGNANT:				
39 C	5	43.7 ± 4.9^{a}	43.6 ± 5.1	$2.08 \pm .07^{d}$
42 C	5	103.8 ± 11.1^{b}	78.1 ± 7.4^{c}	$2.72 \pm .10^{e}$

[a] $P<0.001$, for PGF secretion by cyclic vs pregnant tissues incubated at 39 or 42 C.

[b] $P<0.001$, for PGF secretion by cyclic and pregnant tissues incubated at 39 vs 42 C.

[c] $P<0.001$, for PGE_2 secretion by cyclic and pregnant tissues incubated at 39 vs 42 C.

[*] PGF production by the generating system = 3.8 ± .08 ng/h.

[d] $P<0.001$, for PGF secretion by the prostaglandin generating system co-cultured with endometrial cytosol from pregnant tissues incubated at 39 C vs the generating system alone.

[e] $P<0.01$, for PGF secretion by the prostaglandin generating system co-cultured with endometrial cytosol from pregnant tissues incubated at 42 C vs the generating system alone.

has previously been shown that most PGF and PGE_2 present in culture medium of endometrial explants is derived from de novo synthesis of PG during culture (Thatcher et al., 1984). When incubated at 39 C, endometrial synthesis of PGF was greater (P<0.001) in tissues from cyclic (123.4 ± 8.3 ng/8 h) than in those from pregnant (43.7 ± 4.9 ng/8 h) cows. Synthesis of PGE_2 by endometrium was similar between pregnancy statuses (cyclic, 50.0 ± 3.7 ng/8 h; pregnant, 43.6 ± 5.1 ng/8 h).

Heat stress incubation temperature (42 C) stimulated prostaglandin synthesis by endometrium regardless of reproductive status (Table 5-2). Mean concentrations of PGF detected in endometrial-conditioned culture medium were greater (P<0.001) for heat-stressed tissues from cyclic (182.7 ± 8.0 ng/8 h) and pregnant (103.8 ± 11.1 ng/8 h) cows. Absolute increases in secretion of PGF by heat-stressed endometrium were similar between cyclic (59.3 ng/8 h) and pregnant (60.1 ng/8 h) cows. Mean concentrations of PGE_2 detected in culture medium were 56.8 % greater (P<0.001) for cyclic (78.4 ± 4.3 ng/8 h) and 79.1 % greater (P<0.001) for pregnant (78.1 ± 7.4 ng/8 h) cows.

Inhibition of prostaglandin synthesis by cultured endometrium

Cotyledonary microsomes from parturient cows were utilized as a prostaglandin generating system for the detection of cytosolic inhibitors of prostaglandin synthesis present in endometrium cultured at 39 or 42 C. The synthesis of PGF by cotyledonary microsomes (3.8 ± .08 ng/h) was not altered when microsomes were incubated with cytosol from endometrium of cyclic cows, regardless of incubation temperature (39 C, 3.7 ± .06 ng/h; 42 C, 3.7 ± .05 ng/h). In contrast, synthesis of PGF by the microsomal prostaglandin generating system was decreased 44.7 % (P<0.001) when incubated with cytosol from endometrium of pregnant cows incubated at 39 C (2.1 ± .07 ng/h). The decrease in PGF synthesis was 28.9 % less (P<0.01) with cytosol from endometrium of pregnant cows incubated at 42 C (2.7 ± .1 ng/h). Within the pregnant tissues, incubation at 42 C caused a 35.3 % relative reduction in inhibitory activity compared to the response at 39 C.

Discussion

Lactating cows, maintained under hyperthermic summer conditions were heat stressed effectively as indicated by a substantial increase in respiratory rates and a 2.3 C elevation in rectal temperatures. Such increases in rectal temperature have been associated with decreased pregnancy rates of lactating dairy cows during the summer months (Roman-Ponce et al., 1977; Badinga et al., 1985).

In the present experiment, uterine production of PGF_2 was monitored in the peripheral circulation of thermoneutral and heat-stressed cows by measurement of plasma concentrations of PGFM. Measurement of PGFM in the peripheral circulation provides an accurate estimate of endogenous levels of $PGF_2\alpha$ (Kindahl et al., 1976). For example, estradiol-induced increases in peripheral PGFM concentrations in cyclic or early pregnant cows (Rico et al., 1981; Thatcher et al., 1984, 1986) is accompanied by an increase in $PGF_2\alpha$ in the uterine lumen (Bartol et al., 1981) and by an increase of $PGF_2\alpha$ synthesis within the uterus (Knickerbocker et al., 1986b; Thatcher et al., 1984). Prior to oxytocin challenge, concentrations of PGFM in the peripheral circulation were similar between pregnant and non-pregnant cattle on Day 17 post estrus and were not effected by heat stress. Likewise, basal PGFM concentrations in pregnant and cyclic cattle were not different prior to either estradiol (Rico et al., 1981) or oxytocin (LaFrance and Goff, 1985) treatment on Days 17 to 19 of the estrous cycle. Elevated oxytocin-induced peaks in peripheral concentrations of plasma PGFM were detected in non-pregnant cows, whereas oxytocin-induced PGF synthesis was attenuated in pregnant cows with normal embryos. Data from the present study using endometrial cultures confirm that secretion of $PGF_2\alpha$ by endometrium also is attenuated during early pregnancy in cattle.

Injection of oxytocin induced an acute rapid increase in uterine production of $PGF_2\alpha$ within 5 to 15 min in non-pregnant and pregnant cows with retarded embryos, regardless of environmental treatment imposed. Increases in PGFM concentrations in the peripheral circulation were 3 to 4 fold higher for nonpregnant compared to pregnant cows carrying normal size embryos at Day 17 post estrus. Similar differential increases in concentrations of PGFM in cyclic and pregnant cattle in response to estradiol (Rico et al., 1981) or oxytocin (Oyedipe et al., 1984; Lafrance and Goff, 1985) injections have been reported. Decreased responsiveness of pregnant cows to an oxytocin-induced increase in uterine $PGF_2\alpha$ production, suggests that the conceptus attenuates the release of uterine $PGF_2\alpha$ and further supports the concept of a conceptus antiluteolytic-anti$PGF_2\alpha$ effect during early pregnancy in cattle (Thatcher et al., 1988).

Conceptuses were recovered from 10 of 30 experimental cows, resulting in a Day 17 pregnancy rate of 33.3 %. Furthermore, conceptuses recovered from 4 of 10 pregnant cows were severely retarded in development based on their weights, and appeared to be fragmented and undergoing degeneration. These conceptuses, if allowed to develop in utero, would most likely fail to survive. Therefore, overall fertility (% good quality, viable conceptuses) of cows exposed to an environmental management system during summer heat stress until Day 17 post-estrus was 20 %. This is higher than the pregnancy rate of lactating dairy cows during the summer in Florida (Badinga et al., 1985; Putney et al., 1988b).

Cows with retarded conceptuses responded to oxytocin challenge with a rapid increase in uterine production of PGF$_2\alpha$. Plasma PGFM concentrations and the relative increase above pre-oxytocin levels were similar to those observed in non-pregnant cows. Similarly, Betteridge et al., (1986) reported that recipient cattle which underwent early embryonic death and luteolysis had increased episodic peaks of peripheral PGFM similar to profiles observed in non-pregnant cattle. Thatcher et al. (1984) also speculated that the marked variation in PGFM responses of pregnant cows to an injection of estradiol was related to viability of the conceptus. Conceptuses, retarded in development, may fail to produce biochemical signals in adequate amounts required for attenuating the release of uterine PGF$_2\alpha$. This may compromise successful rescue of the corpus luteum from luteolysis since this event may depend directly upon the appropriate timing and quantity of secretory products produced by the conceptus. For example, infusion of bovine conceptus secretory proteins into the uterine lumen from day 15.5 to day 21 will extend the interestrous interval (Knickerbocker et al., 1986a) and attenuate uterine PGF$_2\alpha$ production in response to an injection of estradiol on Day 18 post-estrus (Knickerbocker et al., 1986c). Since recurring heat stress retards conceptus development in vivo (Biggers et al., 1987), and decreases conceptus protein synthesis and secretion in vitro, particularly bovine trophoblast protein-1, (Putney et al., 1987), heat-stressed retarded conceptuses may be unable to signal their presence to the maternal system and attenuate the release of uterine PGF$_2\alpha$ during establishment of pregnancy. This may compromise lutal lifespan and lead to increased pregnancy failure in cattle exposed to high ambient temperatures.

Heat stress increased oxytocin-induced uterine production of PGF$_2\alpha$ by nonpregnant cows. A heat stress induced responsiveness of cyclic endometrium to oxytocin may be due, in part, to increased availabiltiy of oxytocin receptors because of alterations in endometrial membrane fluidity, or to activation of intracellular signal transduction associated with oxytocin binding to its receptor on the endometrial cell membrane. Heat stress may potentiate oxytocin-induced prostaglandin synthesis in endometrium by activating oxytocin receptor mediated second messengers such as inositol phosphates and diacylglycerol (Calderwood et al., 1987). Heat stress induced phosphoinositide breakdown to inositol phosphates and diacylglycerol act as intracellular second messengers leading to increased mobilization of intracellular Ca^{+2} (Stevenson et al., 1986). Increased intracellular Ca^{+2} concentrations may stimulate endometrial PGF$_2\alpha$ production by activating phospholipase A$_2$, since this enzyme is Ca^{+2} dependent (Brockerhoff and Jensen, 1974).

Heat stress in vitro induced a marked increase in basal endometrial synthesis of PGF and PGE$_2$ by pregnant and cyclic cows. Increased prostaglandin synthetic capacity of endometrium exposed to heat stress also may be due, in part, to heat induced alterations in endometrial cellular membranes resulting in increased mobilization of phospholipids and release of arachidonic acid (Calderwood et al., 1987), which provides substrate for prostaglandin synthesis (Thatcher et al., 1984, Flint et al., 1986). Synthesis of PGF by heat-stressed endometrium of pregnant cows was 43 % lower than that of heat-stressed endometrium of cyclic cows. Attenuated increase in heat-induced synthesis of PGF by pregnant endometrium may be due to the presence of an active intracellular endometrial inhibitor of prostaglandin synthesis (Basu and Kindahl, 1987; Gross et al., 1988a). Although heat stress partially inactivated the prostaglandin inhibitor, its activity was greater in stressed pregnant compared to cyclic endometrium.

Heat stress of pregnant cows had no effect on uterine PGF_2 production. Inability of heat stress, to induce responsiveness of pregnant endometrium to oxytocin may be due, in part, to conceptus induced increases in the activity of the endometrial prostaglandin synthesis inhibitor (Gross et al., 1988c) which may suppress uterine $PGF_{2\alpha}$ production, and as a result, render the endogenous uterine prostaglandin generating system unresponsive to exogenous stimulation by heat stress. In vivo, the conceptus was continuously present during the period of heat.

In summary, synthesis of PGF, in vitro, by endometrium from cyclic cows was greater than that by endometrium from pregnant cows. Similarly, oxytocin-induced increases in peripheral PGFM concentrations were higher for nonpregnant and pregnant cows with retarded embryos than for pregnant cows with normal embryos. These data support the concept of a conceptus induced antiluteolytic-antiPGF$_2$ effect on the pregnant uterus during early pregnancy in cattle. Heat stress increased the oxytocin-induced concentrations of PGFM in non-pregnant cows, and increased synthesis of PGF and PGE$_2$ by endometrial explants from cyclic and pregnant cows. The absolute increases in PGF secretion induced by heat stress was similar between cyclic and pregnant endometrium. However, overall mean response in pregnant tissues was less due to the presence of, at least a partially active prostaglandin inhibitor. Decreased responsiveness of the pregnant uterus to an oxytocin or heat induced secretion of PGF suggests the presence of an active conceptus induced intracellular, endometrial prostaglandin synthesis inhibitory factor that attenuates the responsiveness of the endogenous uterine prostaglandin generating system to exogenous stimulation by heat stress.

CHAPTER 10

GENERAL DISCUSSION

Seasonal periods of high ambient temperature and relative humidity often results in transient infertility in dairy cattle. Although various reproductive processes are affected adversely by an unfavorable thermal environment, early embryonic mortality is the major factor causing summer-time depression of pregnancy rate in cattle. One of the most critical periods in the life of the bovine embryo and one of relatively high mortality occurs during the first three weeks of gestation (Ayalon, 1972; Diskin and Sreenan, 1980). Exposure of dairy heifers to acute heat stress during the early stages of oocyte maturation (see Chapter 2) or to intermittent heat stress during the first 7 days of pregnancy (see Chapters 3 and 6) resulted in extensive morphological abnormalities and developmental retardation and/or arrest of development among stressed embryos.

Exposure of oocytes to high intraovarian temperatures during the early stages of nuclear and cytoplasmic maturation, may result in the failure of embryos to undergo genomic activation at the 8- to 16-cell stage (Newcomb, 1982, Eyestone and First, 1986) and continued development to the compacted morula and expanded blastocyst stages. Embryonic development did not progress beyond the 8- to 16-cell stage by Day 7 or pregnancy in 85 % of embryos recovered from stressed heifers. Heat stress during oocyte meiosis may produce gametes with unbalanced (polyploid) chromosomal composition. Such oocytes are capable of fertilization and may result in embryos with chromosomal abnormalities. Since very few live-born animals with chromosomal anomalies have been reported, the genetically abnormal embryo fails to survive as development progresses and most are eliminated during the first month of gestation, prior to establishment of pregnancy (Williams and Long, 1980).

Rate of embryonic development was slowed considerably in heifers exposed to intermittent heat stress. High oviductal and uterine temperatures of hyperthermic heifers may have directly altered the metabolic activity of stressed embryos, resulting in developmental retardation and a reduced rate of embryonic growth. In vitro heat stress of Day 7 bovine embryos (see Chapter 4) reduces total protein synthetic capacity while enhancing the synthesis of heat shock proteins (hsps). Heat-stress induced suppression of protein synthesis, in vivo, may result in embryonic retardation by possibly delaying or blocking cellular mitotic activity of stressed embryos. Cell cycle arrest and decreased mitotic activity are an important part of the heat shock response in all eucaryotic species so far investigated (Nover, 1982).

A reduced rate of embryonic growth, may result in asynchronous development between the preimplantation embryo and gravid uterine horn. A maternal microenvironment at an inappropriate stage for a delayed embryo may compromise embryo viability and most likely lead to subsequent embryonic death. Bovine embryo transfer studies have demonstrated clearly the critical importance of the state of the uterine environment for viability and development of the embryo (Rowson et al., 1972). Embryo transfer between animals that are not in estrus at the same time (± 1 to 2 days) established that during early pregnancy, embryo development depends upon an appropriate sequence of changes in uterine secretions to provide histotrophic nutrition to the developing conceptus. Deviation from this relationship due to heat-stress induced embryonic retardation or to inappropriate alterations in the composition of uterine secretions may cause abnormal embryo development and lead to increased rates of embryo death.

Heat shock proteins play an essential role in cellular homeostasis and thermotolerance during periods of environmental stress (Loomis and Wheeler, 1980; Li and Werb, 1982). Thermotolerance is closely correlated with the ability of embryos to produce hsps (Muller et al., 1985; Heikkila et al., 1985). By the morula to blastocyst stage of development bovine embryos are able to synthesize hsps in response to heat shock and, as a result, may have acquired some degree of thermotolerance and ability to survive acute increases in uterine temperature as often occurs when cattle are exposed to high ambient temperatures.

Embryo transfer may provide an alternative to artificial insemination to circumvent summer-time depression of pregnancy rate in cattle (see Chapters 5 and 6). Pregnancy rates of lactating cows, exposed to summer heat stress, were increased significantly when embryos were transferred to such cows at Day 7 post estrus. Increased pregnancy rates of recipient cows indicates that the early embryo of cows that are artificially inseminated is extremely sensitive to harmful effects of maternal hyperthermia and fails to survive to Day 21 post-estrus. Embryo transfer may bypass this early developmental period of extreme embryonic thermal sensitivity.

Bovine embryos continue to be sensitive to maternal hyperthermia beyond the period of early cleavage and blastocyst formation (see Chapter 7). In vitro elevation of incubation temperature of Day 17 bovine conceptuses from 39 to 42 C reduces total protein synthetic capacity while enhancing the synthesis of hsps. Reoccuring heat stress retards conceptus development in vivo (Biggers et al., 1987), and decreases conceptus protein synthesis and secretion in vitro, particularly bovine trophoblast protein-1 (Putney et al., 1987, Chapter 7). Heat-stressed retarded conceptuses may be unable to produce antiluteolytic-antiPGF biochemical signals at the proper time (Days 16 to 17 post-estrus) or in adequate amounts required to signal its presence to the maternal system and attenuate the release of uterine PGF in order to maintain CL function. For example, lactating cows, maintained under shade a management system during periods of summer heat stress, which carried retarded conceptuses responded to oxytocin challenge on Day 17 post-estrus with a rapid increase in uterine production of PGF (Chapter 9). Plasma PGFM concentrations and the relative increase above pre-oxytocin levels were similar to those observed in non-pregnant cows.

Exposure of pregnant cows to high environmental temperature and humidity, as often occurs during summer months of the year, may disrupt the balance between conceptus and endometrial biochemical factors responsible for maintenance of pregnancy. Maintenance of luteal function and establishment of pregnancy in pregnant cattle is associated with diminished secretion of PGF during the expected period of luteolysis (Thatcher et al., 1984; see Chapters 8 and 9). Likewise, the release of PGF_2 from the uterus in response to estrogen (Thatcher et al., 1984) and oxytocin (Lafrance & Goff, 1985; see Chapter 9) is attenuated during early pregnancy. Heat stress perturbs this physiological difference in prostaglandin secretion between cyclic and pregnant cattle. Heat stress, in vitro, induced a marked increase in endometrial synthesis and secretion of PGF by endometrial explants from pregnant and cyclic cows at Day 17 post-estrus Oxytocin-induced secretion rates of $PGF_{2}\alpha$ were increased from perifused pregnant endometrium exposed to heat stress and similar to those by cyclic endometrium maintained at thermoneutral temperatures.

Heat stress may increase synthesis of endometrial $PGF_{2}\alpha$ by altering factors which regulate prostaglandin biosynthesis. Exposure to high ambient temperature, in vitro, is accompanied by alterations in membrane lipid composition (Anderson & Parker, 1982) as well as increases in membrane fluidity, phospholipase activity and phosphoinositide turnover (Calderwood et al., 1987). Heat-induced increases in the turnover of membrane phospholipids and release of fatty acids, such as arachidonic acid, may provide substrates for prostaglandin synthesis (Flint et al., 1986).

A heat stress induced responsiveness to oxytocin by endometrium from pregnant and cyclic cows may be due in part to activation of oxytocin receptor mediated second messengers such as inositol phosphates and diacylglycerol (Flint et al., 1986; Calderwood et al., 1987). Heat-stress induced increases in phosphoinositide breakdown to inositol phosphates and diacylglycerol increases intracellular second messengers leading to increased mobilization of intracellular Ca^{+2} (Stevenson et al., 1986). Increased intracellular Ca^{+2} concentrations may stimulate endometrial $PGF_{2}\alpha$ production by activating phospholipase A_2, since this enzyme is Ca^{++} dependent (Brockerhoff and Jensen, 1974).

Synthesis of PGF by heat-stressed endometrium of pregnant cows was lower than that of heat-stressed endometrium of cyclic cows. Attenuated increase in heat-induced synthesis of PGF by pregnant endometrium may be due to the presence of an active endometrial prostaglandin synthesis inhibitor, EPSI, (Basu and Kindahl, 1987; Gross et al., 1988a). Although heat stress partially inactivated the prostaglandin inhibitor (see Chapter 9), its activity was greater in pregnant compared to cyclic endometrium. In vivo heat stress, of pregnant cows on Day 17 of pregnancy had no effect on uterine PGF production (Chapter 9). Inability of heat stress, to induce responsiveness of pregnant endometrium to oxytocin may be due to conceptus induced increases in the activity of EPSI, which may suppress uterine $PGF_{2}\alpha$ production, and as a result, render the endogenous uterine prostaglandin generating system unresponsive to exogenous stimulation by heat stress.

In conclusion, successful preimplantation embryonic development and maintenance of pregnancy represents the culminated results of numerous and complex interactions between the developing conceptus and its mother. Survival of the conceptus and associated maintenance of the corpus luteum are major biological limitations that contribute to the high rate of pregnancy failure that occurs in cattle prior to implantation. In tropical and subtropical areas of the world normal levels of pregnancy failure are exacerbated during seasonal periods of high ambient temperature. Research presented in this dissertation furthers our understanding of the physiological and biochemical processes within the preimplantation bovine embryo and maternal endometrium, affected by heat stress, that contribute to increased embryonic mortality in cattle exposed to high ambient temperatures. From these studies, future management systems can be developed to optimize reproductive performance during seasonal periods of infertility due to heat stress.

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BIOGRAPHICAL SKETCH

Dennis James Putney, son of Robert V. and Pauline E. Putney, was born on March 10, 1959, in Tonawanda, New York. He attended the Dryden Central School system and graduated from Dryden High School in June, 1977. He entered the United States Army in November, 1977, and while in the service attended The University of Texas, at El Paso, as a biology major. He graduated from Cornell University in January, 1982, with a Bachelor of Science degree in Animal Science. His undergraduate Honors thesis was entitled "The Comparative Utilization of Urea by Hamsters and Rats as Affected by Dietary Fiber".

He began his graduate studies at Virginia Polytechnic Institute and State University in March, 1983, under the guidance of Dr. W.E. Beal in the Department of Animal Science. In December, 1984, he received a Master of Science degree in animal science. His masters thesis was entitled "The Effect of Prenatal Androgen Exposure on Sexual Differentiation and Postnatal Growth in Beef Cattle".

During January, 1985, he began a doctoral program of research under the direction of Dr. William W. Thatcher and Dr. Maarten Drost in the Department of Dairy Science, University of Florida, Gainesville. Upon completion of the Doctor of Philosophy Degree, in April, 1988, he will continue his training in reproductive biology in the laboratory of Dr. Eugene D. Albrecht in the Department of Obstetrics and Gynecology, University of Maryland, School of Medicine, in Baltimore, as a postdoctoral fellow.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William W. Thatcher, Chair
Professor of Animal Science
and Dairy Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maarten Drost, Cochair
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Fuller W. Bazer
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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